Modelling Soft Tissue Sarcomas Using Somatic Mouse Genetics

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde

(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Laura Patricia Brandt

aus

Deutschland

Promotionskommission

Prof. Dr. Ian Frew

(Leitung der Dissertation)

Prof. Dr. Bernd Bodenmiller

(Vorsitz der Dissertation)

Prof. Dr. Peter Wild

Prof. Dr. Vartan Kurtcuoglu

Zürich, 2017

SUMMARY

Soft tissue sarcomas are rare mesenchymal tumours accounting for 1% of adult malignancies and are fatal in approximately one third of patients. One of the most aggressive and lethal subtypes of soft tissue sarcomas are angiosarcomas. Angiosarcomas are rare malignancies of endothelial differentiation that account for approximately 1% of all soft tissue sarcomas. They are clinically aggressive and highly metastatic and they show a wide anatomic distribution. Treatment options are limited and the prognosis is poor. Therefore, novel targeted treatments are needed for the improvement of the survival of these patients. A prerequisite for this is a better understanding of the biological mechanisms underlying this tumour type and to define the spectrum of genetic alterations that cooperate to cause the formation of these tumours.

The aim of this study was to employ a lentiviral gene regulatory system (MuLE system) to generate *in vitro* and *in vivo* models that reflect the molecular alterations of human angiosarcoma and to use them for the testing of molecularly-targeted therapies. Mice were systemically injected with MuLE lentiviruses expressing combinations of shRNA against *Cdkn2a*, *Trp53, Tsc2* and *Pten* with or without expression of *H-Ras, PIK3CA* and *c-Myc*. The systemic injection of an ecotropic lentivirus expressing oncogenic *H-Ras* together with the knockdown of *Cdkn2a* or *Trp53* was sufficient to initiate the development of angiosarcoma and/or undifferentiated pleomorphic sarcoma, another aggressive soft tissue sarcoma subtype. None of the other genetic combinations were sufficient to induce tumour formation. Unexpectedly, different mouse strains developed different types of sarcoma in response to identical genetic drivers, implicating genetic background as a major contributor to the genesis and spectrum of sarcomas.

To investigate the effect of the induction of oncogene and tumour suppressor expression *in vitro*, primary murine endothelial cells from the spleen were infected with lentiviruses expressing the aforementioned combinations. These genetically defined cell lines were then used for the testing of the therapeutic effects of a panel of kinase inhibitors. The initial screen revealed that cells expressing oncogenic *H-Ras* together with the knockdown of *Cdkn2a* are more sensitive to PI3K/mTOR pathway inhibitors as well as CHK1 pathway inhibitors. However, more detailed drug-response analyses

revealed that there was no clear tendency for the cells with *H-Ras* and *Cdkn2a* gene alterations to be more sensitive to the inhibitors than those with wild type genes.

The *in vivo* testing of the MEK inhibitor AZD6244 and the dual PI3K/mTOR inhibitor NVP-BEZ235 showed no differences between vehicle and inhibitor-treated animals. We conclude that these therapeutic regimes are not likely to be suitable for the treatment of Ras-driven human angiosarcomas.

In summary, our experiments show that the MuLE system can be used to establish genetically-defined autochthonous mouse tumour models that reflect the human disease.

ZUSAMMENFASSUNG

Weichteilsarkome sind seltene mesenchymale Neoplasien, die 1% aller bösartigen Tumore im Erwachsenenalter ausmachen und bei circa einem Drittel der Patienten tödlich verlaufen. Ein aggressiver und häufig lethal verlaufender Subtyp des Weichteilsarkoms ist das Angiosarkom, welches sich durch seine endotheliale Differenzierung auszeichnet und ungefähr 1% aller Weichteilsarkome repräsentiert. Angiosarkome können am ganzen Körper beobachtet werden. Durch den häufig fodrouyanten klinischen Verlauf und die hohe Metastasierungsrate, sind Angiosarkome nur schwer behandelbar. Die 5-Jahres-Überlebensrate liegt bei 10-12%. Die zurzeit vorliegenden Therapieformen haben keinen wesentlichen Einfluss auf die Überlebensrate. Demzufolge, werden neue zielgerichtete molekulare Therapien dringend benötigt. Voraussetzung dafür ist ein besseres Verständnis der dem Angiosarkom unterliegenden molekularen Mechanismen und die genaue Bestimmung der genetischen Veränderungen, die zu der Entstehung dieser Tumorart führen.

Ziel dieser Doktorarbeit war es, mit Hilfe eines lentiviralen genregulatorischen Systems (MuLE System) *in vitro* und *in vivo* Modelle zu generieren, welche die molekularen Veränderungen des humanen Angiosarkoms widerspiegeln. Diese Modelle sollten anschließend für das Testen zielgerichteter molekularer Therapien verwendet werden. Dafür wurden Mäuse systemisch mit MuLE Lentiviren, welche Kombinationen von shRNAs gegen *Cdkn2a*, *Trp53*, *Tsc2* und *Pten* alleine oder zusammen mit der Exprimierung von *H-Ras*, *PIK3CA* und *c-Myc* enthalten, injiziert. Die systemische Injektion ecotropher, oncogener *H-Ras* und sh*Cdkn2a* oder sh*Trp5*3-exprimierender Lentiviren führte zu der Entwicklung von Angiosarkomen und/oder undifferenzierten pleomorphen Sarkomen. Bei undifferenzierten pleomorphen Sarkomen handelt es sich, genau wie bei Angiosarkomen, um einen hochmalignen Weichteilsarkomsubtypen. Keine der anderen getesteten genetischen Kombinationen war ausreichend um die Entwicklung von Tumoren zu initiieren. Erstaunlicherweise entwickelten unterschiedliche Mausstämme, trotz identischer genetischer Stimuli, verschiedene Sarkomarten. Dies deutet auf eine entscheidende Rolle des genetischen Hintergrunds bei der Tumorentwicklung hin.

Zur Untersuchung der Onkogeninduktion und Tumorsuppressorexprimierung *in vitro* wurden primäre murine Endothelzellen aus der Milz mit Lentiviren, welche die zuvor aufgeführten Kombinationen exprimieren, transduziert. Diese genetisch-definierten Zelllinien wurden anschließend für die Erforschung des therapeutischen Effekts ausgewählter Kinaseinhibitoren verwendet. Erste Screenings zeigten, dass Zellen, welche oncogenes *H-Ras* und sh*Cdkn2a* exprimieren, sensitiver auf PI3K-mTOR und CHK1 Inhibitoren reagieren. Detallierte Dosis-Wirkungs-Analysen konnten dies jedoch nicht bestätigen.

Bei der Ermittlung der therapeutischen Effekte des MEK Inhibitors AZD6244 und des dualen PI3K/mTORs Inhibitors NVP-BEZ235 *in vivo* konnten keine Unterschiede zwischen den mit Trägersubstanz und Inhibitor-behandelten Tieren festgestellt werden. Diese Therapeutika sind daher nicht für die Behandlung von Ras-gesteuerten humanen Angiosarkomen geeignet.

Zusammenfassend konnte gezeigt werden, dass mit Hilfe des MuLE Systems genetisch-definierte, autochthone Mausmodelle, welche die humane Erkrankung widerspiegeln, etabliert werden können.

ACKNOWLEDGEMENTS

First and foremost I would like to thank Ian Frew for giving me the opportunity to work in his lab. I appreciate his guidance during my PhD.

Furthermore, I would like to thank Peter Wild and Bernd Bodenmiller for supporting me as members of my PhD thesis committee with valuable input and help during the annual meetings and for the substantial collaboration over the last year.

Many thanks to Joachim Albers, who has been very patient and supportive with a lot of interesting discussions regarding the progression of the project. This thesis would not have been possible without his work.

I would like to acknowledge all current and former members of the Frew and Wenger lab for their willingness to help and the nice atmosphere.

Thank you to Svende Pfundstein and Nevena Zivanovic for providing their expertise and their contribution to this thesis.

Lastly, I would like to express my gratitude to my friends and most of all my family and Tomas for their continuous support and endless encouragement.

Zurich, January 2017
Laura Brandt

TABLE OF CONTENTS

ABBREVIATIONS

4E-BP1	Eukaryotic translation initiation factor 4E binding protein 1
CAT1	Cationic amino acid transporter 1
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CRISPR	Clustered regularly interspaced short palindromic repeats
DMSO	Dimethyl sulfoxide
ERK	Mitogen-activated protein kinase
GAP	GTPase activating protein
GEF	Guanine nucleotide exchange factor
HIF1α	Hypoxia inducible factor 1α
KSHV	Kaposi's sarcoma-associated herpes virus
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
MFH	Malignant fibrous histiocytoma
MMLV	Moloney murine leukemia virus
MSC	Mesenchymal stem cell
mTOR	Mammalian target of rapamycin
MuLE	Multiple lentiviral expression
NF1	Neurofibromatosis type I
PDK1	Phosphoinositol-dependent kinase 1
pEEC	Primary endometrial epithelial cell
PI3K	Phosphatidylinositol 3-kinase
PI(4,5)P_2	Phosphatidylinositol (4,5)-bis-phosphate
PI(3,4,5)P_3	Phosphatidylinositol (3,4,5)-tris-phosphate
PIK3CA	Phosphatidylinositol (4,5)-bis-phosphate 3-kinase catalytic subunit α
PKB	Protein kinase B (also called AKT)
pMSEC	Primary mouse spleen endothelial cell
PTEN	Phosphatase and tensin homolog
PTP	Protein tyrosine phosphatases
PTPRB	Protein tyrosine phosphatase, receptor type B
RAF	Mitogen-activated protein kinase kinase kinase
RB	Retinoblastoma
RTK	Receptor tyrosine kinases

rtTA	Reverse tetracycline-controlled transactivator
S6K	S6 kinase
SRB	Sulforhodamine B
TCGA	The Cancer Genome Atlas
TRE	Tetracycline response element
TSC1/2	Tuberous sclerosis protein 1 and 2
UPS	Undifferentiated pleomorphic sarcoma
VEGF	Vascular endothelial growth factor
VE-PTP	Vascular endothelial protein tyrosine phosphatase
WHO	World Health Organization

1 INTRODUCTION

The goal of this thesis was to better understand the genetic events contributing to the development of soft tissue sarcomas, especially angiosarcomas. This thesis focused on identifying functional genetic interactions between some of the most commonly altered genes in human angiosarcomas, such as *TP53*, *CDKN2A*, *MYC*, *HRAS*, *PIK3CA*, *PTEN* and *TSC2*. Current knowledge about the signalling pathways contributing to angiosarcoma formation and targeted therapeutic options are reviewed in the following sections.

1.1 Soft tissue sarcoma

Soft tissue sarcomas are rare and diverse mesenchymal malignancies which account for approximately 1% of all adult cancers (1). In Europe the incidence rate ranges between 3.3 and 4.7 per 100,000 per year with a five-year relative survival of 58% (2). The incidence increases with age and tumours are observed in both male and female patients. Soft tissue sarcomas can occur at almost any anatomic site with the most common sites being the extremities, trunk, retroperitoneum or head and neck region. The World Health Organisation (WHO) has defined over 100 different benign and malignant soft tissue sarcomas subtypes named after the tissue which they most closely resemble (3).

Most soft tissue sarcomas arise spontaneously, but there are several well described heritable genetic syndromes that are associated with an increased risk for sarcoma development (4). Those which account for the largest number of cases are Li-Fraumeni syndrome, which is caused by *TP53* mutations (5), hereditary retinoblastoma (RB) (6) and neurofibromatosis type I (NF1) (7). Two known sarcoma-associated environmental risk factors are ionizing radiation, especially in the form of radiotherapy used for cancer treatment (8), and vinyl chloride exposure, which is used extensively in the plastic industry (9). Common soft tissue sarcomas that develop after radiation therapy are angiosarcomas and undifferentiated pleomorphic sarcomas (UPS) (10,11).

1.1.1 Molecular characterization of soft tissue sarcoma

Based on molecular characteristics, soft tissue sarcomas can be divided in two categories: low-grade sarcomas with simple karyotypes, such as chromosomal translocations, and high-grade sarcomas with more complex genetic profiles. Genetically complex sarcomas are clinically more aggressive than karyotypically simple sarcomas. Despite the genetic diversity of the subtypes, comparison of these two groups provides a useful framework for considering the genetic mechanisms which underlie sarcomagenesis (12-14).

Soft tissue sarcomas with simple karyotypes account for approximately one third of all sarcomas and include Ewing's sarcoma (15), alveolar rhabdomyosarcoma (16) and synovial sarcoma (17). These tumours typically arise *de novo* and commonly contain tumour-specific chromosomal translocations of which most encode aberrant transcription factors. Less common are chimeric protein tyrosine kinases and autocrine growth factors. The cytogenetic abnormality is present at the initiation of sarcoma development and retained throughout the clonal evolution (13,18). Sarcomas with chromosomal translocations are much more common in children than adults (19).

Genetically complex sarcomas can arise from a benign lesion and progess to a genetically instable, malignant tumour. Examples for this are the progression of benign leiomyomas to malignant leiomyosarcomas (20). However, most high-grade sarcomas are believed to arise *de novo* without preceding benign lesions. Examples for genetically complex, high-grade sarcomas are UPS, leiomyosarcoma and angiosarcoma (21), which all have a high prevalence of *TP53* and *RB1* mutations or deletions (22,23). High-grade, genetically complex sarcomas show complex chromosomal alterations, but recurrent chromosomal translocations are absent. Their underlying genetic mechanism involves disturbances in genes involved in the p53 pathway, including *TP53* mutation, *CDKN2A* deletion and *MDM2* amplification (13,24,25).

Molecular analysis of sarcoma subtypes has indicated that previous classifications, based on the site of the tumour and the histo-pathological appearance are less important than the underlying molecular alterations. This is especially important for the development and clinical application of new molecularly-targeted sarcoma therapies,

which are urgently needed to improve the survival of these patients (26,27). So far, standard treatment options are surgical resection, radiotherapy, and chemotherapy. Patients with inoperable, advanced or metastatic disease have a poor prognosis which has not improved much over the past few decades (28).

1.2 Undifferentiated pleomorphic sarcoma (UPS)

UPS, formerly known as malignant fibrous histiocytoma (MFH), is a malignant neoplasm of uncertain origin that arises both in soft tissue and bone (3). MFH was first described in 1961 by Kauffman and Stout. They described it as a tumour rich in histiocytes with a storiform or cartwheel-like growth pattern (29). With advances in immunohistochemistry, the phenotype of this tumour was shown to be more closely aligned with a fibroblast than a histiocyte (30). In 2002, the WHO declassified MFH as a formal diagnostic entity and renamed it as UPS (3). Despite discrepancies in definition criteria, there is agreement that tumours, which by immunohisto-pathological analysis show a lack of tissue-specific differentiation and a combination of storiform and/or pleomorphic areas as well as by electron microscopy show fibroblastic/myofibroblastic features, can be classified as UPS (31). UPS account for approximately 5% of adult soft tissue sarcomas and represent one of the most common types of high-grade soft tissue sarcoma (32). UPS is characteristically a tumour of late adult life with a peak incidence rate in persons between the ages of 50 and 70 years (33). Approximately two thirds of UPS patients are male with higher rate of incidence among Caucasians compared to African Americans or Asians. The tumours occur most commonly in the lower extremity, especially the thigh, followed by the upper extremity. The majority of UPS are high-grade lesions with a local recurrence rate, a metastatic rate and a five-year survival of around 30%, 35% and 50% respectively. The most common site of metastasis is lung, followed by bone and liver (34-36).

The etiology of UPS is a constant topic of debate. It has been suggested that UPS a group of de-differentiated sarcomas which share a common morphology but originated from different cell types. UPS may have multiple cells of origin with a convergent undifferentiated endpoint. This shared morphological pattern could be the result of common underlying genetic changes rather than a common cell of origin (37,38).

On the contrary, current data implicates mesenchymal stem cells (MSCs) as the common cell of origin of UPS tumours. MSCs were first described and isolated in 1980 as adherent fibroblast-like appearing cells within bone marrow aspirates (39). MSCs have the ability to self-renew and differentiate into a variety of mesenchymal lineages, such as chrondocytes, osteoblasts and adipocytes. Oncogenic transformation of MSCs at various time points during differentiation can result in discrete histological sarcoma subtypes. Transformation at early time points of soft tissue differentiation would result in less differentiated tumours, while the transformation at later time points of differentiation would cause the development of more differentiated sarcomas (40-42). Gazziola *et al.* were the first who associated UPS with undifferentiated MSCs. Gene expression analysis was utilised to profile and compare bone marrow-derived MSCs with primary undifferentiated sacromas, leiomyosarcomas with smooth muscle cells and fibrosarcomas with fibroblasts. They demonstrated that there was the same level of similarity between all three pairs (43). Furthermore, Matushansky *et al.* have shown that the gene expression profile of UPS overlaps with the profile of uncommitted MSCs prior to differentiation (44). Nonetheless, there is still a lack of general consensus whether UPS is a distinct tumour entity or the most undifferentiated endpoint of sarcomas that might initially have showed more differentiation.

1.2.1 Molecular characterization of UPS

Due to the rarity of studies on genetic alterations in UPS, the molecular alterations underlying the development of this heterogeneous malignancy are not completely understood. Recently, a comprehensive genomic characterization of 48 UPS samples was performed by the Cancer Genome Atlas (TCGA) with the provisional data now available publicly. Exploration of the data with the help of cBioportal (Fig.1.1) (45,46) revealed that, like other genetically complex sarcomas, UPS show a high prevalence of p53 pathway alterations (23). *TP53* alterations have been identified in 65% of the samples and *CDKN2A* loss in 23%. *RB1* was deleted in 33% of the patients. *MYC* amplifications were found in 6% of UPS lesions. Deregulated PI3K/AKT/mTOR signalling has been implicated in complex karyotype soft tissue sarcoma development and progression (47). The analysis of the TCGA UPS data confirmed genetic alterations in *PIK3CA*, *PIK3R1* and *PIK3R2*, *PTEN*, *mTOR*, *RHEB* and *TSC1* and *TSC2* in a small percentage of UPS tumours. Furthermore, several growth factor

receptors were altered. Although the only MAPK pathway alterations reported in the TCGA dataset were *NF1* alterations, *HRAS* and *KRAS* mutations have been identified in human UPS tumours as well (48-50).

So far, standard treatment options are surgical resection combined with radiotherapy and/or chemotherapy. However, a high precentage of recurrence or metastatic spread remain problematic. In light of the genetic complexity of UPS, the molecular analysis of the tumours rather than the histo-pathological classification is crucial for the improvement of treatment options (27,35).

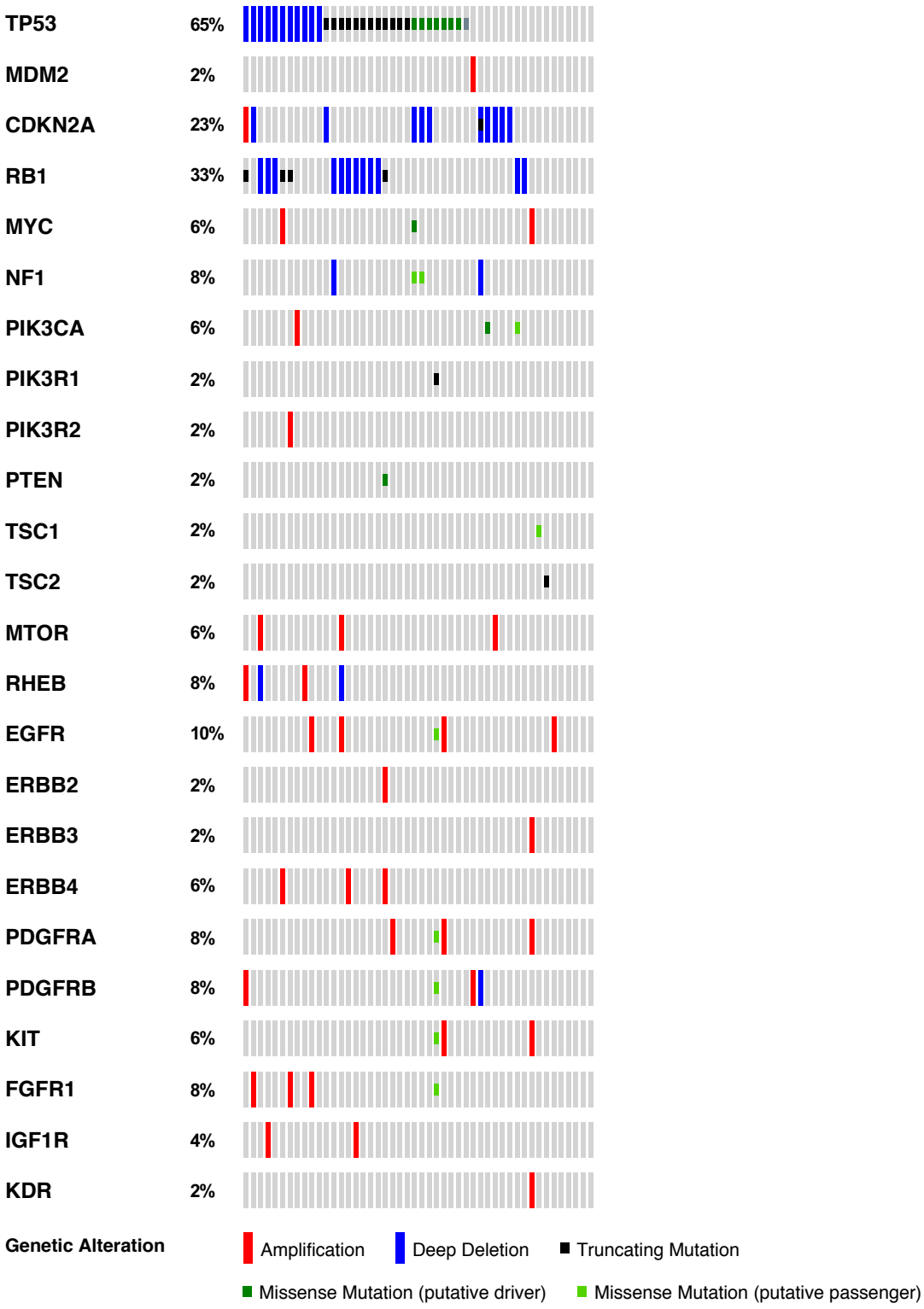


Figure 1.1. Co-occurence of mutations in UPS samples. Oncoprint of genetic alterations identified in 48 UPS samples from the TCGA database (provisional).

1.3 Angiosarcoma

A less frequent but equally clinically aggressive subtype of high-grade soft tissue sarcomas are angiosarcomas. These tumours represent rare malignancies of mostly endothelial but also lymphatic differentiation that account for approximately 1% of all soft tissue sarcomas. Angiosarcomas are the most malignant subtype of a larger group of vascular neoplasms. Other members include benign hemangioma and intermediate hemangioendothelioma and Kaposi's sarcoma-associated herpes virus (KSHV)-derived tumours (3,51). Although incidences of angiosarcoma are observed in any age-group, it is more common in older patients with a similar distribution between sexes. They exhibit a high rate of lymph node and lung metastases with 5-year survival rate less than 30%. Angiosarcomas can arise in the skin, connective tissue or viscera and they show a wide anatomic distribution. The common sites of angiosarcoma development listed according to frequencies of occurences are head and neck regions, followed by the breast, extremities, trunk, liver, heart and spleen (52-55). Angiosarcomas are present as infiltrative, multinodular hemorrhagic masses with morphologies varying from spindle to epithelioid neoplasms. Extensive haemorrhage is a characteristic of many tumours. The majority of angiosarcomas a high-grade tumours characterized by cells of high nuclear grade displaying mitotic activity (56,57). Angiosarcomas mostly express the typical endothelial markers: CD34, CD31, FLi1, ERG and von Willebrand factor, however progressive tumour de-differentiation can cause the loss of these markers in some cases (58-61).

Most cases of angiosarcoma arise spontaneously. Known risk factors include chronic lymphoedema, radiation and exposure to toxic chemicals. Stewart-Treves syndrome or lymphoedema-associated angiosarcoma, is a rare complication of chronic lymphoedema mainly related to breast cancer (62). Another known risk factor is radiotherapy, which can cause the development of secondary angiosarcoma, especially in the breast with a peak incidence 5-10 years after treatment (63). Various chemicals are also associated with angiosarcoma development, particularly within the liver. Hepatic angiosarcoma may arise after exposure to vinyl chloride, which is used extensively in the plastic industry (64). Furthermore, thorium dioxide, which has been used in radiology, is also associated with hepatic angiosarcoma development (65). Other chemical carcinogens that promote angiosarcoma development include arsenic, radium and anabolic steroids (66).

1.3.1 Molecular characterization of angiosarcoma

Due to its rarity, only few comprehensive studies of genetic alterations found in angiosarcoma patients have been reported. Genetic mutations and amplifications of *VEGF*, *MDM2*, *TP53*, *CDKN2A*, *KRAS* and *MYC* have been described in angiosarcoma patients (67-70). *MYC* gene amplifications are commonly found in a subset of secondary, radiation-induced angiosarcomas (71). A recent publication reported that the majority of genetic alterations were found in the p53 and MAPK pathways (Fig. 1.2). *TP53* was mutated in 35% of the lesions and *CDKN2A* lost in 26%. 53% of angiosarcomas displayed MAPK pathway activation, and harboured genetic activating mutations in *KRAS*, *HRAS*, *NRAS*, *BRAF*, *MAPK1* or inactivating mutations in *NF1* and *PTPRB1* (72,73). Furthermore, alterations in the PI3K/AKT/mTOR pathway have been identified in a small percentage of patients (73-75).

Although these recent studies have been very helpful in providing starting points for new avenues of research, the molecular and cellular mechanisms underlying angiosarcoma development are still not fully understood.

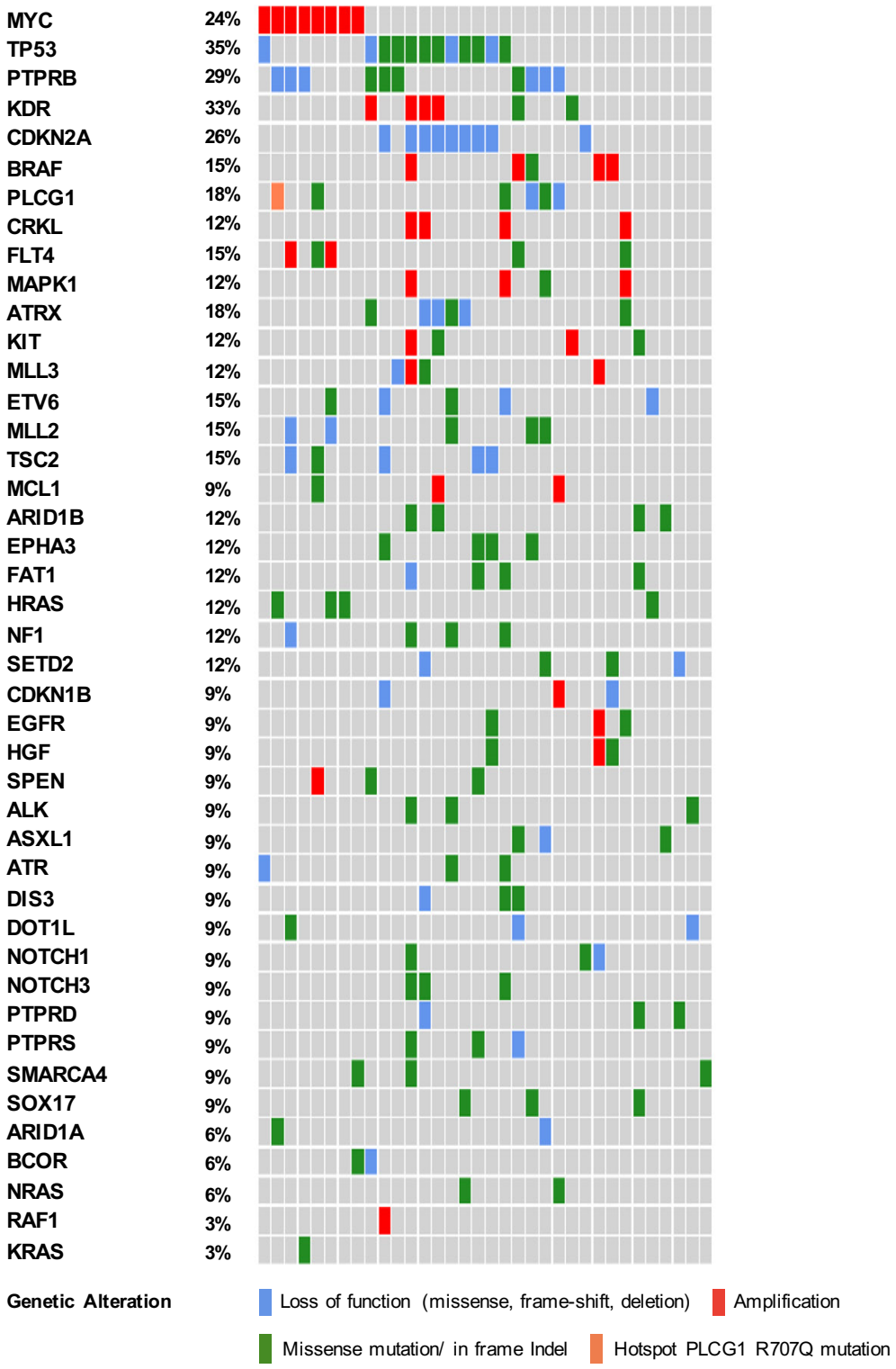


Figure 1.2. Co-occurence of mutations in angiosarcoma samples. Oncoprint of genetic alterations identified in 34 angiosarcoma samples. Adapted from (73).

1.4 Angiogenesis and VEGF signalling

Angiosarcoma is an aggressive soft tissue sarcoma of presumed endothelial origin that is commonly thought to be driven by aberrant angiogenesis.

Angiogenesis is the process of new blood vessel formation from existing blood vessels. In the adult, it is tightly regulated and only transiently turned on during physiological processes such as the female reproductive cycle and wound healing (76). Without a vascular supply of oxygen and nutrients, tumour growth is limited to a certain size (1-2 mm3). In response to a variety of stimuli, such as oncogenic *H-Ras* (77) or hypoxia (78), an expanding tumour exploits angiogenesis for its benefits. Intratumoural hypoxia causes the activation of an angiogenic switch to increase the blood supply by provoking an increased expression of hypoxia inducible factor 1α (*HIF1*α), which activates the transcription of genes which encode pro-angiogenic factors, such as the vascular endothelial growth factor (VEGF) (79,80). VEGF in turn can initiate a downstream signalling cascade and thereby stimulate the growth of new blood vessels. In addition to supplying the tumour with oxygen and nutrients, the newly formed vessels also provide an escape route for the tumour cells to metastasise. Due to aberrant and imbalanced angiogenic signalling, the new tumour vessels are unorganised, immature and leaky (81,82).

In many solid tumours, VEGF expression correlates positively with angiogenesis (83). Five VEGF glycoproteins have been identified so far, with VEGF-A being the most important member (hereafter referred to as VEGF). VEGF binds in an overlapping pattern to the two receptor tyrosine kinases (RTKs), VEGFR-1 and -2. While VEGFR-1 primarily serves as a negative regulator of VEGF signal transduction, VEGFR-2 transduces all normal and pathological effects of VEGF (84-86). Gene targeting of *Vegf* and *Vegfr2* in mice both result in early embryonic lethality due to defective vascular development (87-89). Binding of VEGF to VEGFR-2 induces receptor dimerization, kinase activation and autophosphorylation of the tyrosine residues. Autophosphorylated residues create docking sites for signal transducers, which activate signalling cascades, such as the mitogen-activated protein kinase (MAPK) or phosphatidylinositol 3-kinase (PI3K) signalling pathway, which cause the induction of cellular processes such as endothelial cell survival, proliferation, migration and lumen formation (Fig. 1.3) (90,91).

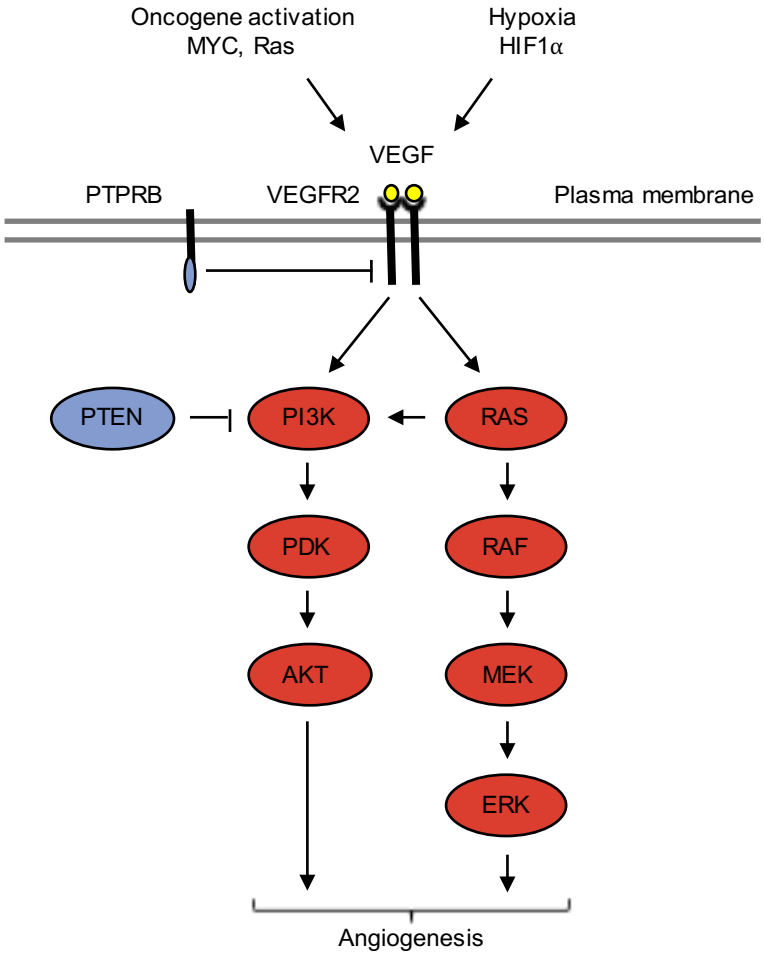


Figure 1.3. Simplified schematic of VEGF signalling. Oncogene activation or hypoxia cause the induction of VEGF expression. VEGF binds to VEGFR2 which can activate different downstream signalling cascades, such as the PI3K and MAPK signalling pathway. PTPRB can dephosphorylate and thereby inactivate VEGFR2. The activation of these signalling cascades stimulates the formation of new blood vessels.

Kinase activity is tightly regulated via several mechanisms. One example is regulation by protein tyrosine phosphatases (PTPs). The vascular endothelial PTP (VE-PTP in mouse and PTPRB in human) is specifically expressed in endothelial cells, where one of its functions is the association and dephosphorylation of VEGFR2 (92-94). Inactivation of the *ve-ptp* gene in mice results in vascular malformations, leading to embryonic death at E11 (95).

Several studies have identified aberrant VEGF signalling as an important driver of angiosarcoma development (67,96). Immunohistochemical analysis of 34 human angiosarcomas revealed VEGF-A overexpression in 94%, VEGFR-1 in 94% and VEGFR-2 in 65% of the samples. Patients who expressed low or no VEGFR-2 showed a significantly unfavorable prognosis (97). Gene expression profiling and sequencing

of 42 human angiosarcoma samples identified VEGFR-2 upregulation and mutations in 10% of the samples (98). The overexpression of VEGF in an immortalised endothelial cell line caused the development of angiosarcoma and upregulated VEGFR-1 and -2 expression *in vivo* (99). Moreover, Ghahremani *et al.* reported that the hetero- and homozygous deletion of VEGF in a angiosarcoma mouse model resulted in a substantial rescue from endothelial cell transformation (100).

Another gene reported to be mutated in angiosarcoma is the endothelial phosphatase PTPRB, a signal transducer of tyrosine kinases. PTPRB mutations were often identified in tumours that showed MYC amplification, a biomarker of radiation-induced secondary angiosarcoma. 15 out of 39 angiosarcomas (38%) were reported to harbor at least one driver mutation in angiogenesis signalling genes (72). These findings suggest that upregulation of the VEGF signalling pathway may play a significant role in angiosarcomagenesis.

1.5 p53 signalling

As in other genetically complex soft tissue sarcomas, case reports and genomic analysis have reported abnormalities in the p53 signalling pathway including *CDKN2A* inactivation, *TP53* mutations and *MDM2* overexpression in spontaneous angiosarcoma development (67,74,101-108).

The p53 tumour suppressor protein plays a key role in cell cycle regulation, apoptosis and senescence (109). Intact p53 signalling is essential for cancer prevention, consistent with a high tumour incidence observed in *Trp53* null mice and *TP53* heterozygous Li-Fraumeni patients (110,111). Approximately 50% of human cancer patients carry p53 alterations and in the majority of the remaining 50%, p53 signalling is inactivated by upregulation of p53 inhibitors, such as MDM2, or by the downregulation of p53 cooperators, such as ARF (112-114). The increased predisposition of tumour development in the absence of p53 is due to the accumulation of genetic alterations and failure to eliminate these defective cells (115).

p53 acts as a cellular stress sensor and can be activated in response to DNA damage, hypoxia and mitogenic or oncogenic activation (116). p53 activation leads to protein

stabilization, sequence-specific DNA-binding and transcriptional target gene activation (117). In undamaged cells, p53 is highly unstable and present at very low concentrations. The major negative regulator of p53 is the MDM2 proto-oncogene that acts as an ubiquitin ligase which targets p53 for proteasomal degradation (118,119). Phosphorylation of p53 by various kinases, including ATM, ATR and CHK1 and 2, after DNA damage reduces its binding to MDM2, which causes decreased proteasomal degradation and in turn increased p53 levels. Stabilized p53 binds to the DNA in a sequence-specific manner and activates or represses its target genes (120). The majority of cancer-associated mutations occur in the DNA-binding domain of p53 (112).

The importance of MDM2 as a negative regulator of p53 is demonstrated by the lethal effect during embryonic development of *Mdm2* knockout mice. The early mortality of these mice can be rescued by co-deletion of *Trp53*. The overexpression of MDM2 in many cancers is often sufficient to inactivate p53 withour further mutation (121).

Tumours without *TP53* alterations often show indirect inactivation of p53 function, through the overexpression of MDM2 or the inactivation of the cell cycle inhibitor $p14^{ARF}$ ($p19^{ARF}$ in mice) (112). $p14^{ARF}$ is encoded by the cyclin-dependent kinase inhibitor 2A (*CDKN2A*) locus on human chromosome 9p21, which also encodes another tumour suppressor gene named $p16^{INK4A}$ (122). *In vivo* knockout of $p16^{INK4A}$ and $p19^{ARF}$ or both genes causes the development of a spectrum of spontaneous tumours (123-126). The $p16^{INK4A}$ protein inhibits the activity of cyclin D-dependent CDK4 and 6 to prevent the phosphorylation of the RB tumour suppressor, which controls the progression through G1 phase. The $p14^{ARF}$ tumour suppressor can bind to MDM2 to inhibit p53 degradation and thereby promote p53 activation. The inactivation of p53 causes the induction of $p14^{ARF}$ expression, while the inactivation of RB causes $p16^{INK4A}$ induction. Therefore, *CDKN2A* inactivation recapitulates the effect of RB and p53 co-deletion, and equips the cells with an enhanced proliferative potential (Fig. 1.4) (127,128).

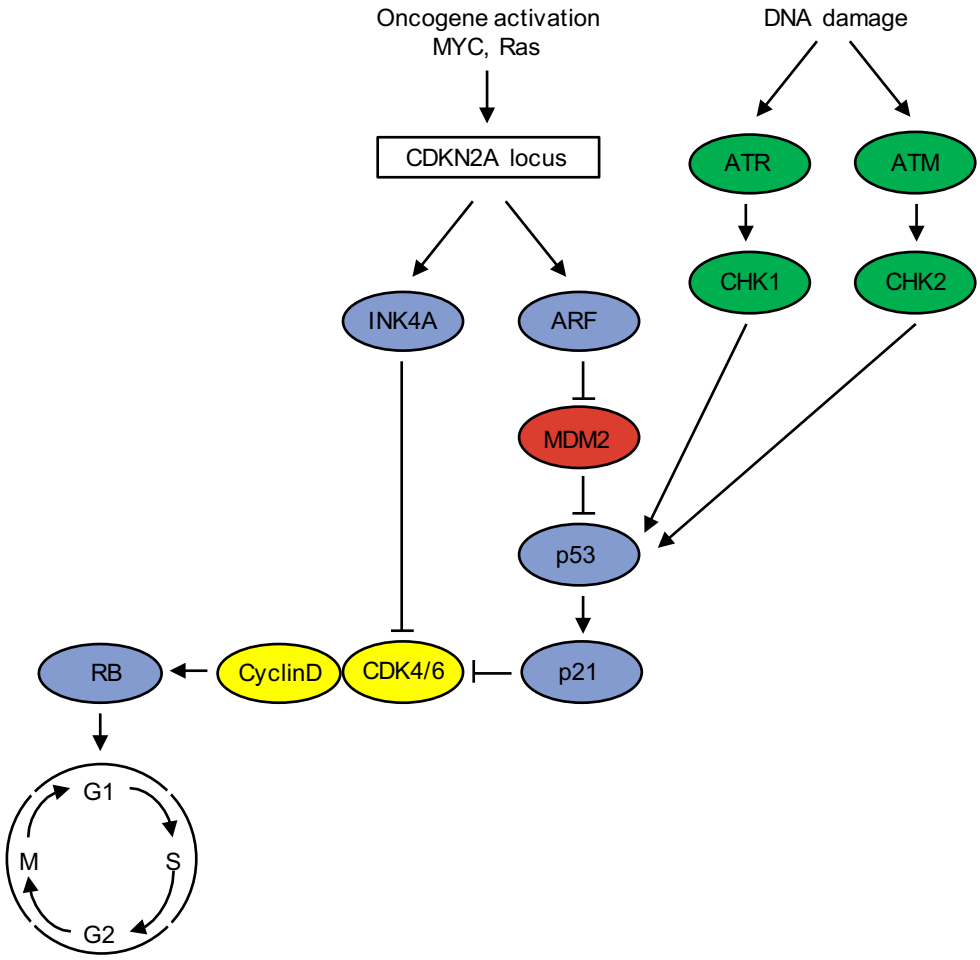


Figure 1.4. Simplified schematic of p53 signalling. Oncogene activation causes the induction of the tumour suppressors INK4A and ARF. ARF increases p53 stability by inhibition of the negative regulator of p53, MDM2, thereby inhibiting p53 degradation. DNA damage causes the activation of ATR/CHK1 and ATM/CHK2. CHK1 and 2 can directly phosphorylate p53 and thereby stabilize it. p53 can induce the expression of the cyclin-dependent kinase inhibitor, which inhibits the activity of cyclin D-dependent CDK4 and 6 to prevent the phosphorylation of the RB tumour suppressor, which controls the progression through G1 phase. INK4A can directly inhibit CDK4/6.

The genomic analysis of 34 angiosarcomas showed that the most frequently detected genetic alterations were mutations in *TP53* in 12 tumours (35%) and the loss of *CDKN2A* in 9 tumours (26%) (73). The loss of CDKN2A expression is associated with a significantly worse prognosis (129). Angiosarcomas arising in rats and humans after vinyl chloride exposure, have been shown to carry *TP53* mutations (130). Moreover, elevated MDM2 levels and p53 mutation are correlated with increased VEGF expression, suggesting that alterations in the p53 signalling pathway may drive sarcomagenesis by upregulating VEGF expression (67). These observations suggest that alterations in the p53 signalling pathway contribute to angiosarcoma development.

1.6 Ras and MAPK signalling

It has been reported that 53% of angiosarcomas displayed MAPK pathway activation and harboured genetic activating mutations in *KRAS*, *HRAS*, *NRAS*, *BRAF*, *ERK* or inactivating mutations in *NF1* (73).

Ras signalling is involved in cellular proliferation, survival, differentiation and angiogenesis as well as metastases. Ras proteins are small GTPases that serve as transducers by converting signals from RTKs and spread them along several distinct downstream signalling pathways (131). *RAS* genes are the most frequently mutated oncogenes in human tumours. There are three closely related RAS proteins in humans, namely H-, K- and N-RAS (132). Although highly similar and widely expressed in adult tissues and tumours, *K-RAS* mutations are much more common in human cancers than *N-RAS* or *H-RAS* (133). This could be due to the unique properties the K-RAS protein, which favour oncogenic transformation. For example, only *K-RAS* confers stem-like properties to certain cell types (134). Moreover, mice lacking *K-Ras* die during embryogenesis whereas mice lacking *N-Ras* or *H-Ras* are viable (135). Another reason for the unbalanced amount of *K-RAS* mutations in comparison to *N-RAS* and *H-RAS* mutations is the preferential expression of specific RAS isoforms in different tumour-bearing organs (136). RAS activation alone is however often not sufficient to initiate cellular transformation. The expression of oncogenic *RAS* in primary cells causes permanent G1 arrest and cooperation of another oncogene or the incativation of tumour suppresors, such as p53 or p16 is required for oncogenic transformation (137-139). These findings extended the concept of multistep cancer progression.

The GTPase RAS functions as a molecular switch cycling between two distinct conformational states, active when GTP is bound and inactive when GDP is bound. In response to upstream signals, Ras proteins are converted from their GDP state to their GTP state by guanine nucleotide exchange factors (GEFs), thereby activating RAS. GTPase activating proteins (GAPs), such as Neurofibromin (NF1), convert RAS-GTP back RAS-GDP, thereby negatively regulating RAS protein function (140). The most common somatic mutant forms of RAS are resistant to GAP-mediated hydrolysis and are locked permanently in the GTP-bound active state. As a consequence multiple RAS-dependent downstream effector pathways are activated (141).

One of the key signalling pathways stimulating cellular proliferation and differentiation is the mitogen-activated protein kinase (MAPK) signalling pathway. Growth factor-induced activation of RAS activates RAF (MAPK kinase kinase), which phosphorylates and thereby activates MEK (MAPK kinase) which in turn phosphorylates ERK (MAPK). ERK phosphorylates a variety of substrates, including other protein kinases, as well as gene regulatory proteins in the nucleus. The resulting changes in gene expression and protein activity cause changes in cellular behaviour (142). Activating mutations in the different components of the RAS-MAPK pathway play an important role in oncogenesis. E.g. RAF mutations are commonly identified in human tumours, such as melanoma and colorectal cancer, and they do not overlap with RAS (143).

Alterations in *K-RAS* have been frequently found in sporadic and vinyl chloride-induced angiosarcomas (69,105,107). Immortalised murine endothelial cells form benign hemangiomas *in vivo*, the addition of activated *H-Ras* causes the development of rapidly growing, poorly differentiated angiosarcomas (77). Moreover, H-Ras activation in an endothelial cell line causes the upregulation of ERK and AKT signalling. Upregulated ERK caused increased proliferation, while AKT controlled vascular morphogenesis. Both AKT and ERK caused increased endothelial cell survival (144).

1.7 PI3K signalling

Alterations in the PI3K/AKT/mTOR pathway have been identified in a small percentage of angiosarcoma patients (73-75).

The PI3K signalling pathway stimulates cell survival and growth. Members of this pathway include the positive regulators phosphoinositide 3-kinase (PI3K), protein kinase B (PKB, also called AKT), mammalian target of rapamycin (mTOR) and as well as the negative regulators phosphatase and tensin homolog (PTEN) and tuberous sclerosis protein 1 and 2 (TSC1/2) (145). There are numerous genetic alterations that result in increased PI3K/mTOR signalling in human tumours (146). The PIK3CA gene, which encodes the PI3K catalytic isoform p110α, is the second most frequently mutated oncogene after RAS and its negative regulator PTEN is among the most commonly mutated tumour suppressor genes (147,148). Moreover, cancer cells with

normal PI3K and PTEN levels show alterations in other genes of the PI3K signalling pathway, such as RAS and AKT activation and loss of TSC1 or 2 (149).

Growth factor-activated RTKs and GTPases recruit and activate PI3K via their regulatory subunit or adapter molecules. PI3K lipid kinases can be divided into three groups (classes I, II and III). The most important class in cancer is class I. Class I PI3Ks are receptor-regulated phosphatidylinositol (4,5)-bis-phosphate ($PI(4,5)P_2$) kinases which generate phosphatidylinositol (3,4,5)-tris-phosphate ($PI(3,4,5)P_3$). The catalytic subunit of class I PI3Ks consists of four isoforms, termed p110α, p110β, p110γ and p110δ that are constitutively bound to a regulatory subunit (150,151). Among the four class I isoforms of the p110 catalytic subunit of PI3K, only PIK3CA, which encodes p110α, is frequently mutated in human tumours (147). Around 80% of the activating PIK3CA mutations are found in two hotspots in the helical domain and one hotspot in the kinase domain (152). Activation of p110α in the mouse heart results in increased cell size which caused an increased heart size (153). Several mouse models were developed to investigate the role of PIK3CA in tumourigenesis. E.g. inducible, tissue-specific expression of mutant PIK3CA in mammary epithelial cells causes the development of adenosquamous carcinoma or adenomyoepithelioma in mice (154).

The lipid phosphatse PTEN negatively regulates PI3K by dephosphorylating the lipid product $PI(3,4,5)P_3$ to $PI(4,5)P_2$. Impaired PTEN function causes $PI(3,4,5)P_3$ accumulation and in turn activation of downstream signalling. Homozygous *Pten* deletion is embryonically lethal, but heterozygous $Pten^{+/-}$ mice develop tumours in multiple tissues (155-157). Loss of PTEN function is a common event in many human tumours. Despite its many functions, the lipid phosphatase function has been shown to be the major driving force in human tumour development and progression (158).

$PI(3,4,5)P_3$ can bind to proteins that contain pleckstrin homology domains, such AKT and phosphoinositol-dependent kinase 1 (PDK1). The binding promotes AKT phosphorylation at Thr 308 by PDK1. This primes AKT for phosphorylation at Ser 473 by mTOR complex 2, which fully activates AKT serine/threonine kinase activity. Fully active AKT mediates numerous cellular functions including angiogenesis, growth, survival, protein synthesis and apoptosis (Fig. 1.5) (159,160).

Activated AKT phosphorylates and inactivates the negative regulator tuberous sclerosis protein 2 (TSC2), which destabilizes TSC2 and disrupts its interaction with TSC1. An inactive TSC1-TSC2 complex cannot inhibit mTOR complex 1. Active mTOR complex 1 phosphorylates the eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) and S6 kinase (S6K), thereby promoting protein synthesis and cellular proliferation (161,162). TSC1 and 2 proteins are so named because mutations in either of the TSC1 or 2 tumour suppressor genes cause the genetic disease tuberous sclerosis, which is characterized by the development of a variety of benign neoplasms, called hamartomas (163). In mice, homozygous deletion of either *Tsc1* or *2* is embryonically lethal, while heterozygous mice are prone to tumour development in various organs (164,165).

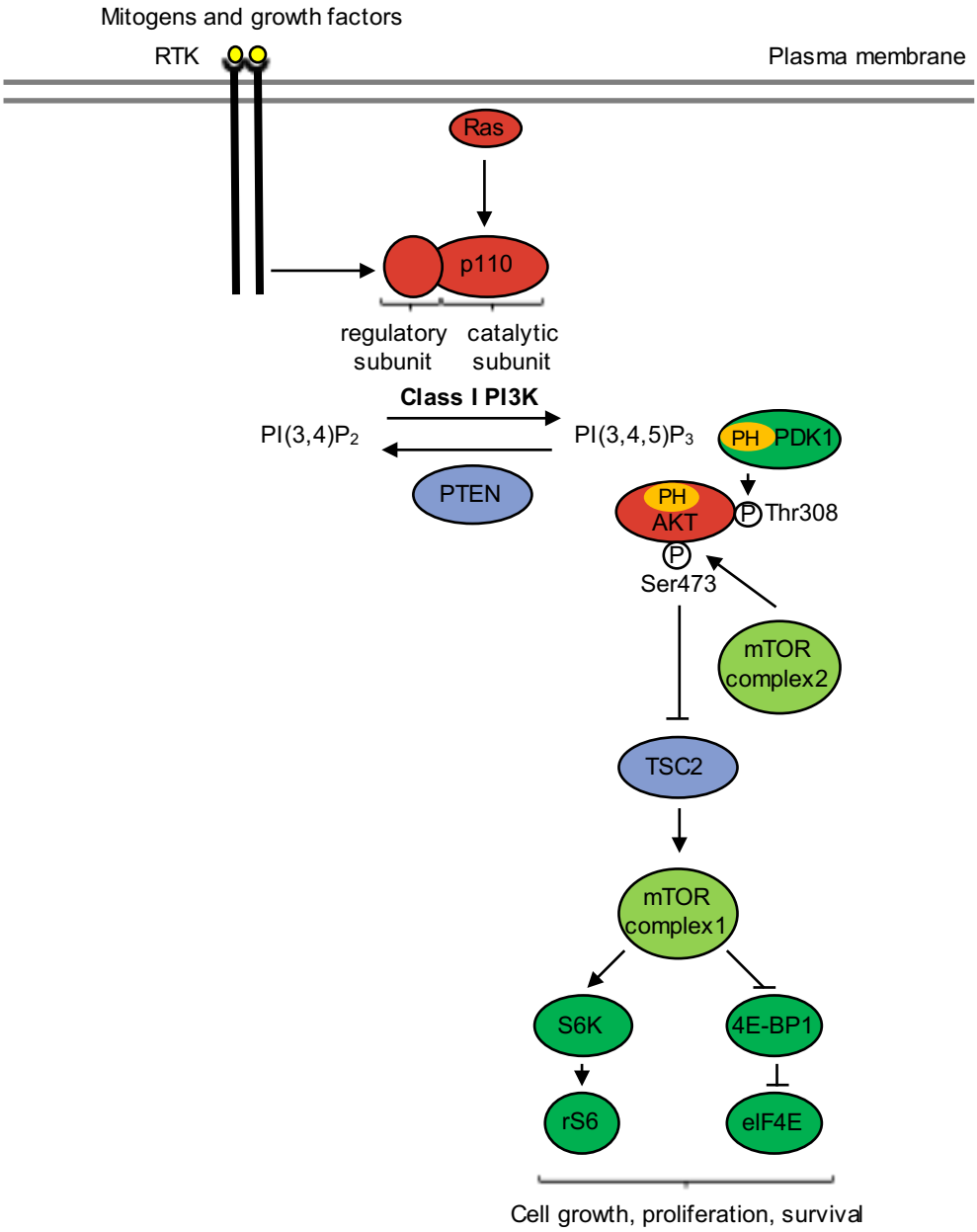


Figure 1.5. Simplified schematic of class I PI3K signalling. PI3Ks are RTK or GTPase-activated, $PI(4,5)P_2$ kinases that generate $PI(3,4,5)P_3$. $PI(3,4,5)P_3$ can bind to the PH domain of AKT and PDK1. This allows the partial activation of AKT by phosphorylation of Thr308 by PDK1. The additional phosphorylation on Ser473 by mTOR complex2 causes the full activation of AKT, thereby causing the activation of AKT target genes, which regulate cellular growth, proliferation and survival. AKT phosphorylation of the tumour suppressor TSC2 causes its inactivation. TSC2 in ist active form can activate mTOR complex1, which in turn phosphorylates 4E-BP1 and S6K.

Human angiosarcoma tumours show infrequent alterations in the PI3K/mTOR signalling pathway, however, because of the rarity of this tumour type, studies regarding the incidence rate of genetic alterations in the PI3K/mTOR signalling pathway of angiosarcoma patients were mostly based on small genetic studies and single case reports. Single case reports revealed loss of heterozygosity of *TSC2* in an

uterine angiosarcoma as well as *PTEN* mutation in a human hepatic angiosarcoma (70,75). Another study showed decreased *PTEN* expression in 41% of bone angiosarcoma patients (129). However, no *PTEN* alterations were identified in 62 soft tissue angiosarcoma patients. Nonetheless, 42% of the patients showed overexpression of phosphorylated ribosomal protein S6 kinase (p-S6K) and/or phosphorylated 4E-BP1 overexpression, suggesting frequent activation of the PI3K/mTOR pathway (74). The overexpression of AKT in an immortalised murine endothelial cell line, resulted in the development of benign vascular lesions (166), further supporting the notion that activation of this signalling pathway may contribute to the pathogenesis of at least some cases of angiosarcomas.

1.8 MYC

Amplification of *MYC* is commonly found in secondary, radiation-induced angiosarcoma (71).

MYC (c-MYC) is a highly regulated and multifunctional proto-oncogenic transcription factor. Homozygous *Myc* mutation in mice is embryonically lethal (167). Several mouse models show the involvement of *Myc* in tumourigenesis and its oncogenic function (168). MYC functions as a master transcriptional regulator of a wide array of target genes and its concentrations need to be tightly regulated in order to avoid oncogenic signalling (169). In tumour cells, MYC expression can be deregulated directly due to gene amplification, chromosomal translocation and polymorphisms in the enhancer region or indirectly due to constitutive activation of upstream signalling pathways (170-175).

MYC is unable to bind to DNA alone and requires a binding partner to bind DNA effectively. A protein named MAX oligomerises with MYC to form heterodimers which are able to bind DNA. This interaction is crucial for MYC-dependent gene regulation (176).

MYC amplification is used as a biomarker of secondary radiation-induced angiosarcomas. Its increased expression in the majority of radiation-induced angiosarcomas suggests that MYC amplification may play an important role in the

development of these tumours (71,177-179). However, a recent study reported MYC gene amplification and protein overexpression in primary, non-radiation-associated cutaneous human angiosarcomas. In this study, MYC gene amplification and protein overexpression were not correlated with the clinical outcome (180). Another group reported variable levels of MYC expression in primary human angiosarcomas with high-grade tumours showing significantly higher MYC expression levels than low-grade tumours (181). Genomic analysis of 34 human angiosarcomas revealed that MYC amplifications are generally mutually exclusive of TP53 alterations and CDKN2A loss (73). These studies collectively argue that MYC is likely to be important for the formation of angiosarcomas.

1.9 Models of angiosarcoma

Genomic studies have revealed a large collection of genetic alterations which are present in human tumours (170,182,183). These alterations either confer a clonal growth advantage to the cell, or are biologically neutral and do not contribute to tumour development (184). The genomic characterization of angiosarcomas necessitates the need for functional genetics to determine which combinations of genetic alterations dictate which tumour phenotype and to develop new *in vitro* and *in vivo* angiosarcoma models for accompanying target discovery (185,186).

Endothelial cell lines isolated directly from angiosarcoma patients are clearly valuable for studies of the molecular biology of the disease. Human cutaneous angiosarcoma-derived cell lines include AS-M (187), ISO-HAS (188) and HAEND (189) as well as a murine-phenotypic angiosarcoma cell line, named ISOS-1, from a transplanted human angiosarcoma into immunocompromised SCID/beige mice (190). All the cell lines show aberrant p53 expression. Typical endothelial cell characteristics include CD31, CD34, VEGFR1 and von Willebrand factor expression, induced expression of cell adhesion molecules and tube formation capacity on Matrigel. However, the angiosarcoma-derived cell lines lost some of these characteristics, such as tube forming capacity and the subcutaneous injection into immunocompromised mice caused the development of de-differentiated tumours (187-192).

Since angiosarcoma-derived cell lines represent end-stage disease and have presumably accumulated several mutations that caused the malignant transformation of the cells, as well as passenger mutations which did not contribute to tumour development, they are not very suitable for modelling earlier stages of endothelial cell transformation. Furthermore, serial passaging of the cells can introduce additional alterations that do not reflect the primary tumours. To overcome these limitations, the modelling of cooperative genetic events which initiate angiosarcomagenesis needs to be based on the genetic manipulation of primary tissue-specific cells. Therefore, genetically engineered mouse models represent a more suitable approach.

The earliest established angiosarcoma models include mice treated with toxic chemicals (193-195). The high incidence of *Ras* mutations in these chemically induced tumours is consistent with human angiosarcoma (196,197).

The most frequent genetic alterations present in human angiosarcomas are alterations in the p53 signalling pathway. Several *in vivo* mouse studies showed the involvement of loss of function of the p53 tumour suppressor in angiosarcoma development (100,198-200). In addition, the *in vivo* deletion of *Cdkn2a* in mice lead to the development of lesions which recapitulate human angiosarcoma, however, only 30% of the mice displayed angiosarcomas within 100 days (201).
Alterations in the PI3K/AKT/mTOR pathway have been identified in a small percentage of patients (73-75) and disruption of the *Tsc1* or *2* tumour suppressors, which negatively regulate the pathway, induced the formation of angiosarcomas in mice (164,165,202,203). The *in vivo* endothelial cell-specific deletion of *Tsc1* caused the development of lymphangiosarcoma and increased VEGF expression in tumour cells (204). Furthermore, overexpression of activated AKT in murine endothelial cells resulted in vascular malformations in mice (166).

Another report showed that the *in vivo* deletion of *Notch1* resulted in the development of hepatic angiosarcomas with a penetrance of 86% at 50 weeks after gene deletion (205,206), although genetic alterations in the Notch pathway, have not been reported in human angiosarcomas.

Although these studies have been helpful in uncovering aspects of sarcomagenesis, there is limited understanding of the interactions between cooperating genetic alterations and the initiating molecular events in existing animal models are largely uncharacterized, and their relevance to human angiosarcomas is uncertain.

1.10 Therapeutic implications

Angiosarcomas belong to the category of genetically complex sarcomas and are highly aggressive tumours. Local recurrences develop in about one fifth of patients and patients with inoperable, advanced or metastatic disease have a poor prognosis which has not improved much over the past few decades (207,208). Rare tumours present major clinical and investigative challenges. Current clinical knowledge of angiosarcoma is based on single case reports and few retrospective case series, where only two published series include more than 100 patients (209,210). Age >70 years, size of the primary lesion and presence or absence of metastases are the most useful determinants of treatment options (211,212).

So far, standard treatment options for local disease are surgical resection and due to the high risk of local recurrence, adjuvant radiotherapy. However, complete surgical resection is the best hope for cure, and radiotherapy is if possible avoided because of the risk of radiation-induced angiosarcomas (53,213). Cytotoxic chemotherapy remains the standard of care for most locally advanced and metastatic angiosarcomas. Regimens include doxorubicin (214-216), ifosfamide (217), gemcitabine (218-220), paclitaxel (221-223), dacarbazine (224), imatinib (225), cisplatin (226,227) and cyclophosphamide (228). Unfortunately, treatment with cytotoxic chemotherapy shows no significant advances in patient survival (208,209,229)

Cellular proliferation and survival often involve signalling through the interconnected MAPK and PI3K/mTOR signalling pathways. Both pathways are normally activated by a growth factor, such as VEGF, binding to a RTK, but may also be activated by downstream events. Because RTKs and their ligands are frequently overexpressed in soft tissue sarcomas, they are thought to represent attractive therapeutic targets. A broad range of RTK inhibitors have been developed in recent years, although, thus far, the response rates in non-translocation-associated soft tissue sarcomas have been

mixed (230).

Upregulation of the VEGF signalling pathway has been suggested to play a significant role in angiosarcoma development. Different strategies have been designed to target the VEGF signalling pathway (231). VEGFR-directed therapies, such as the humanized monoclonal VEGF-neutralizing antibody bevacizumab (Avastin) and the RTK inhibitor sorafenib (BAY43-9006) are associated with approximately 15% response rates in primary and ionising radiation-induced angiosarcomas (232-234), with these responses perhaps associated most clearly with VEGFR2 mutations (98). The treatment of two angiosarcomas with the RTK inhibitor sunitinib (SU11248) did not show prolonged patient survival (235).

The finding of PI3K/mTOR pathway alterations in angiosarcoma patients suggests that this molecular subset of patients might benefit from PI3K and mTOR inhibitors, this is currently being tested in clinical trials for soft tissue sarcoma patients. Elevated VEGF levels in hemangiomas can be reduced by the mTOR inhibitor rapamycin, suggesting a potential link between TSC/mTOR signalling and vascular tumours (236). Furthermore, mTOR inhibition by rapamycin or VEGF blockade effectively abolished vascular tumour development and growth in a *Tsc1* driven lymphangiosarcoma mouse model (204).

Aurora kinases represent a family of serine/threonine kinases required for progressive stages of mitosis and cell division. Dysregulation of Aurora kinases has been reported in a variety of carcinomas, but little data is available on their role in soft tissue sarcoma treatment (237). The treatment of an angiosarcoma with the Aurora A kinase inhibitor alisertib (MLN8237) in combination with cytotoxic chemotherapy caused a partial response (238).

The drop in the cost of personal genome sequencing may alter the clinical and therapeutic course for cancer patients. It is becoming technically possible to guide treatment by the analysis of the patients' genomic alterations (239,240). Furthermore, molecular analysis of sarcoma subtypes has indicated that previous classifications, based on the site of the tumour and the histo-pathological appearance are less important than the underlying molecular alterations driving sarcomagenesis. This is

especially important for the development and clinical application of new molecularly-targeted sarcoma therapies (26,27).

1.11 MuLE system

The afore-mentioned studies demonstrate the usefulness of genetically engineered mice for modelling vascular neoplasias. However, transgenic mouse models also have several limitations caused by the lack of availability of appropriate genetic alleles and the feasibility and time requirements of combining multiple genetic alterations in a single mouse in a tissue-specific manner (241).

Somatic genetic engineering represents an alternative approach for cancer modelling. One example, generated in our laboratory, is a powerful novel system which allows high throughput genetic manipulations in primary cells *ex vivo* and in mouse tissues *in vivo.* This published system, termed multiple lentiviral expression (MuLE) system, utilises lentiviral-mediated delivery of multi-cistronic gene regulatory cassettes to simultaneously cause the overexpression, gene knockdown, Cre-mediated gene deletion, or clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9-mediated gene mutation of multiple genes via a single infection (242). Cloning of these vectors is extremely simple and rapid due to the use of MultiSite Gateway cloning technology (243). MuLE vectors are also marked with either drug resistance genes, fluorescent proteins or luciferase to enable the rapid engineering and monitoring of tumours formed from primary cells in cell culture or formed directly following injection *in vivo* into mouse tissues. For biosafety reasons, MuLE vectors are packaged with an ecotropic viral envelope protein (MLV virus) which restricts viral tropism to rodent cells (244).

Intramuscular injections of MuLE lentiviruses expressing oncogenic *H-Ras*^{G12V} together with combinations of knockdowns of the tumour suprressors *Cdkn2a*, *Trp53* and *Pten* allowed the generation of three murine soft tissue sarcoma models. This example demonstrates that somatic genetic engineering in adult mouse tissues can be used for the rapid generation of genetically-defined autochthonous tumours (242).

1.12 Aims

The utility of existing genetically engineered angiosarcoma mouse models for systematic pre-clinical testing of new therapeutic approaches are limited by unpredictable timing and in some instances by failure to recapitulate the genetics which underlie human angiosarcomas. All models in which tumours formed in a reasonable timeframe (less than 6 months) were generated by crossing two or more transgenic mouse strains, requiring time-consuming and expensive breeding programs.

In this thesis we developed a new mouse genetic approach using the MuLE lentiviral gene regulatory system (242) to functionally test the contributions of some of the most commonly altered human candidate driver oncogenes and tumour suppressor genes, namely by altering the expression of the mouse homologues of the human *TP53, CDKN2A, MYC, HRAS, PIK3CA, PTEN* and *TSC2* tumour suppressor genes, to the formation of angiosarcoma in the mouse.

We aimed to generate a panel of monitorable, genetically-defined angisarcoma mouse models which reflect these genetic mutations. These *in vivo* models could then be used in combination with *ex vivo* studies to identify and test appropriate therapies specific for these combinatorial mutations. This combined approach aimed to ultimately facilitate the development of more personalised treatments for angiosarcoma patients.

2 RESULTS

2.1 Oncogenic *H-Ras*^{G12V} expression plus knockdown of *Cdkn2a* using ecotropic lentiviral vectors induces high-grade endometrial stromal sarcoma

Manuscript in preparation

Oncogenic *H-Ras*^{G12V} expression plus knockdown of *Cdkn2a* using ecotropic lentiviral vectors induces high-grade endometrial stromal sarcoma

Laura P. Brandt[1,2], Joachim Albers[1], Tomas Hejhal[1], Peter J. Wild[3], Ian J. Frew[1,2,4,5].

[1] Institute of Physiology, University of Zurich, Zurich, Switzerland
[2] Zurich Center for Integrative Human Physiology, University of Zurich, Zurich, Switzerland
[3] Department of Pathology and Molecular Pathology, University Hospital Zurich, Zurich, Switzerland
[4] BIOSS Center for Biological Signalling Studies, University of Freiburg, Freiburg, Germany
[5] Center for Translational Cell Research, Clinic of Internal Medicine I, Medical Center - University of Freiburg, Freiburg, Germany

Address for correspondence:
Ian Frew
Center for Translational Cell Research
Medical Center - University of Freiburg
Breisacher Straße 115
79106 Freiburg
Germany

Phone: +49 (0) 761 270 71831
Email: ian.frew@uniklinik-freiburg.de

ABSTRACT

The uterine corpus represents the most common site for tumour development in the female genital system. Uterine neoplasms are categorised as epithelial, mesenchymal, mixed epithelial-mesenchymal or trophoblastic tumours. In this study we employed a mouse genetic approach using the MuLE lentiviral gene regulatory system to functionally test the ability of ecotropic lentiviruses to model epithelial and mesenchymal uterine malignancies *ex vivo* and *in vivo*. We discovered that MuLE lentiviruses efficiently infect uterine stromal cells but not endometrial epithelial cells when injected into the uterus of cycling, pseudopregnant or ovarectomized mice. Consistent with this cellular infection spectrum, we show that intra-uterine injection of ecotropic MuLE viruses expressing oncogenic *H-Ras*^{G12V} together with knockdown of *Cdkn2a* induce high-grade endometrial stromal sarcomas, establishing this approach as an efficient method of generating autochthonous mouse models of uterine sarcomas.

INTRODUCTION

Early-stage uterine tumours are associated with an excellent prognosis after surgical treatment. However, for patients with advanced, metastatic or recurrent uterine cancer, limited therapeutic options are available (1-3). To improve understanding of these diverse diseases it would be desirable to have a series of representative mouse models that reflect not only the different histological types of uterine tumours but also the underlying molecular genetics of each of these different tumour types. In recent years there has been some progress towards this goal.

The most prevalent tumours of the female reproductive system are carcinomas of the endometrium, which are classified in a dualistic model based on histological appearance and molecular differences (4,5). Type I endometrioid tumours account for 80% of endometrial malignancies and are characterised by a relatively high survival rate, due partly to their frequent early detection following abnormal uterine bleeding. Several studies report that *PTEN* mutations occur early in the development of type I tumours (6-10). In terms of generating rapid, flexible and accurate mouse models of this disease, it has been shown that the injection of adenoviruses expressing Cre into the uterine lumen of adult *Pten*$^{fl/fl}$ mice caused the development of uterine carcinomas (11). Tumour onset and severity can be accelerated by combining *Pten* deletion with *K-Ras* activation (12). This combination of germline-based generation of Cre-regulatable genetic alleles plus viral-based delivery of Cre to somatic endometrial cells represents one approach to speed up the generation of different molecular forms of endometrial carcinoma. Type II endometrial tumours are more common in older, non-obese women and, despite their rarity, have a worse outcome due to their highly invasive and metastatic nature. They comprise three different subtypes: serous, clear cell and undifferentiated carcinomas, each representing approximately 5-10% of all endometrial carcinoma cases (13). Type II tumours are associated with both overlapping and distinct sets of genetic alterations to those found in type I tumours. They exhibit extensive somatic copy number alterations. In contrast to type I tumours, type II tumours are frequently characterised by early mutations in *TP53* (14-19). The deletion of the *Trp53* gene in the mouse endometrium results in the formation of all histological subtypes of type II disease after 14-16 months (20).

Uterine mesenchymal tumours are derived from the soft tissue of the uterine corpus, which comprises endometrial stroma, smooth muscle and blood vessels. Mesenchymal tumours account for 3% of uterine tumours (21). Uterine sarcomas are broadly classified into leiomyosarcomas, accounting for 60% of cases, endometrial stromal sarcomas, and undifferentiated sarcomas. These categories are defined by the presence of specific molecular alterations as well as by tumour morphology and prognosis (22). Leiomyosarcomas are composed of cells which demonstrate smooth muscle differentiation. The majority of uterine leiomyosarcomas occur sporadically. Commonly found alterations include *MED12* and *HMGA2,* which represent independent genetic events (23). Patients with germline mutations in fumarate hydratase have an increased risk for developing uterine leiomyosarcomas as well as uterine leiomyomas (24). Copy number loss of the 10q chromosomal region, which contains the *PTEN locus*, has been detected in human leiomyosarcomas and is associated with an aggressive clinical behaviour (25). Moreover, it has been shown that the *in vivo* deletion of *Pten* in smooth muscle cells promotes abdominal and retroperitoneal leiomyosarcomagenesis (26).

Endometrial stromal sarcomas are composed of cells resembling the endometrial stroma and are far less frequent than smooth muscle tumours, such as leiomyosarcomas. They are characterized by chromosomal translocations, most commonly involving the zinc finger genes *JAZF1* and *SUZ1* (27-31). Undifferentiated uterine sarcomas represent a very rare and aggressive category of uterine tumours. Considering their low incidence rate, there is little to no information about molecular alterations found in these tumours. They are diagnosed by exclusion after elimination of other high-grade uterine tumours with a sarcomatous component (32,33). However, again using the combined germline genetics plus viral delivery of Cre approach, injection of an adenovirus expressing Cre-recombinase into the uterus of mice harbouring conditional alleles of oncogenic *K-Ras* and *Trp53* caused the development of undifferentiated pleomorphic sarcoma (34,35).

In order to accelerate the process of generating genetically-complex autochthonous mouse models of human tumours we recently developed the MuLE lentiviral gene regulatory system, (36) that facilitates combinatorial somatic genetics in cultured cells and *in vivo* in mouse tissues. As a proof of principle, we showed that this system could

be employed to generate new mouse models of muscle-derived undifferentiated sarcomas that harbour alterations in multiple signalling pathways (36). In the current study we investigate whether the MuLE system could also be applied to the setting of modelling of different forms of uterine tumours. We show that the MuLE system can be used to model endometrial sarcomas but does not appear to be easily applicable to modelling of uterine carcinomas.

MATERIALS AND METHODS

Mice

SCID/beige mutant mice (C.B-17/CrHsd-Prkdc^{Scid}Lyst^{bg-J}) and C57BL/6JRccHsd mice were obtained from Envigo. B6.Cg-Gt(ROSA)Sor$^{tm14(CAG-tdTomato)Hze}$/J mice were obtained from the Jackson Laboratory. Vasectomized RjOrl:SWISS male mice were obtained from Janvier labs. Pax8-rtTA;LC1 mice (37), were obtained from Prof. Carsten Wagner, University of Zurich. For doxyclycline experiments, 6-8-week old offspring of intercrosses between Pax8-rtTA;LC1 and B6.Cg-Gt(ROSA)Sor$^{tm14(CAG-tdTomato)Hze}$/J mice were exposed to doxycycline (0.5 mg/ml) in drinking water supplemented with 5% sucrose for 5 days. Afterwards animals were kept on normal drinking water for another week. Mouse experiments were approved by the Veterinary Office of the Canton of Zurich under the licence 137/2013.

Ovarectomy

Adult, cycling female B6.Cg-Gt(ROSA)Sor$^{tm14(CAG-tdTomato)Hze}$/J mice were anaesthetized with isoflurane and the ovaries were exposed. Both uterine horns were ligated and the ovaries were removed. 2-week following surgery lentiviruses were injected into the uterus.

Intrauterine viral injections

Approximately 4-week old female mice were anaesthetized using isoflurane and the uteri were exposed by making a small incision. 10µL of concentrated lenti- and adeno-viruses expressing different genetic manipulations were injected into one uterine horn with a 30G insulin syringe. The animals were euthanised 1-6 months after injection depending on the experiment. The uteri were harvested, fixed in 10% formalin, paraffin-embedded and cut in 5µm thick sections. For the identification of the Cre-infected cells, B6.Cg-Gt(ROSA)Sor$^{tm14(CAG-tdTomato)Hze}$/J uteri were fixed in 4% paraformaldehyde for 4-6h, transferred to 30% sucrose overnight, embedded in OCT and cut in 5µm thick sections.

Allograft studies

For allograft experiments, SCID/beige mice were anaesthetized by the inhalation of isoflurane and $1x10^6$ cells resuspended in 50% Matrigel (BD, no. 354230) were injected subcutaeously. *In vivo* imaging as well as a caliper were used to follow tumour development over time.

***In vivo* imaging studies**

Noninvasive *in vivo* fluorescence and bioluminescence imaging was performed using the IVIS Spectrum (Perkin Elmer) together with the Living Image software (version 4.4). Mice were anaesthetized by using 2.5% isoflurane. During imaging, the isoflurane levels were reduced to 1.5% All fluorescence measurements were performed in epi-fluorescence mode. For bioluminescence imaging, mice were injected subcutaneously with 150 mg/kg D-luciferin (Caliper, no. 122796) and imaged 15 min after injection. For the quantification of the total radiant efficiency, a region of interest was drawn around the mouse and the total radiant efficiency was automatically detected.

Isolation and maintenance of pEEC

We used previously published protocols (38,39) with minor modifications for the isolation of primary endometrial epithelial cells from mice. 5-10 3-week-old C57BL/6 mice were sacrificed by cervical dislocation and uterine horns were removed, dissected in Hanks Balanced Salt Solution (HBSS, ThermoScientific) and cut open lengthwise. The fragments were then pooled and incubated with 0.5% Trypsin (Sigma Aldrich)/ 2.5% pancreatin (Thermo Fisher Scientific) in HBSS for 60 min at 4°C and 60 min at 22°C. Following transfer to ice-cold HBSS, digested uteri were vortexed for 30 sec to release epithelial cells. Uterine tissues were filtered through a 40µm nylon mesh and epithelial cells were collected and centrifuged at 1.000 x*g* for 5 min. Cells were then resuspended in basal medium containing DMEM/F12 (Sigma Aldrich) supplemented with 1 mmol/l HEPES (Thermo Fisher Scientific) and 1% penicillin/streptomycin (Sigma Aldrich). Cells were mechanically disrupted by pipetting until clumps of cells are observed under the microscope. Cells were then seeded in basal medium supplemented with 5ng/ml EGF (PeproTech), 1mg/ml insulin (Sigma Aldrich),

0.55mg/ml transferrin (Sigma Aldrich), 0.5µg/ml sodium selenite (Sigma Aldrich) (BIE) and 0.5% dextran-coated charcoal-stripped FCS (Biochrom AG) and plated into cultures dishes. Cells were cultured in BIE plus 0.5% stripped FCS in an incubator at 37°C with saturating humidity and 5% O_2.

Immunohistochemistry

Immunohistochemical analysis was performed on formalin-fixed paraffin sections after antigen retrieval (5 min at 110°C in 0.1M citrate buffer pH6). The antibodies used in this study were anti-CD10 antibody (1:2000, PA5-47075, Thermo Fisher Scientific), anti-CD31 antibody (1:200, ab28364, abcam), anti-CYCLIN D1 antibody (1:40, SP4, Thermo Fisher Scientific), anti-DESMIN antibody (1:100, D1033, Sigma-Aldrich), anti-MYOD1 antibody (1:100, M3512, Dako), anti-MYOGENIN antibody (1:500, M3559, Dako), anti-ALPHA SMOOTH MUSCLE ACTIN antibody (1:5000, ab5694, abcam) and anti-VIMENTIN antibody (1:500, D21H3, Cell Signaling).

RESULTS

Establishment of a primary endometrial epithelial cell culturing system

To study the ability of the MuLE system to model uterine malignancies, we first focused on the most common uterine tumour type, endometrial carcinoma. We established a murine endometrial epithelial cell culture model to allow us to study endometrial tumour development *ex vivo.* Primary endometrial epithelial cells (pEECs) were isolated by digestion of dissected uterine horns in trypsin and pancreatin. Freshly isolated cultures showed almost uniform postive staining for E-CADHERIN, ß-CATENIN and CYTOKERATIN-7 and, with the exception of a small number of cells, negative staining for VIMENTIN, establishing the relative purity of the epithelial isolation (Fig. 1A). Next, we optimised cell culture conditions to allow the proliferation of these cells. The addition of 0.5% charcoal-stripped FCS to the medium allowed expansion of the cells into larger clusters in which cells maintained their epithelial cobblestone morphology. If FCS was not added the cells maintained their epithelial morphology but did not expand and passaging them was not possible (Fig. 1B). Based on these results, we decided to culture the cells with 0.5% FCS.

The extended replicative life span which results from the inactivation of the p53 pathway is thought to represent an important event in the multistep process of endometrial carcinoma development. Given this genetic link to human endometrial carcinogenesis and additionally since pEECs fall into senescence with ongoing cultivation on plastic dishes (40), we tried to immortalise them by using primary epithelial cultures prepared from *Trp53*$^{fl/fl}$ uteri. *Trp53* was deleted *ex vivo* by treating the cells with a Cre-expressing adenovirus. Additionally, we infected pEECs from wildtype mice with a MuLE vector expressing a miR-30-based shRNA against *Trp53* together with antibiotic resistance. Genetic deletion or knockdown of *Trp53* allowed the cells to be expanded and passaged, but passaging still caused a change in cellular morphology to a more fibroblast-like appearance (Fig. 1C), meaning that these cultures do not represent a system that can be used for long-term studies of endometrial epithelial cells.

We next sought to determine whether primary endometrial epithelial cells could be readily infected by MuLE viruses. For these experiments we isolated endometrial

epithelial cells from uteri of Pax8-rtTA; LC1; ROSA26-lox-stop-lox tdTomato mice (37,41). PAX8 is a transcription factor which is essential for the development of the female genital tract, including luminal and glandular endometrial epithelial cells (42). In this mouse model, the reverse tetracycline-controlled transactivator (*rtTA*) is expressed under the control of the Pax8 promotor (*Pax8-rtTA*). In the presence of doxycycline, *rtTA* can bind to a tetracycline response element (*TRE*) which leads to the expression of Luciferase and Cre, specifically in endometrial epithelial cells, which induces the expression of tdTomato (Fig. 2A). Following the administration of female mice with doxycycline to activate Cre-mediated recombination, uteri were harvested and Pax8-driven tdTomato expression in glandular and luminar epithelial cells was confirmed by *in vivo* imaging (Fig. 2B) and microscopy. We isolated pEECs from the uteri of these mice and infected them with ecotropic MuLE lentiviruses expressing GFP to label infected cells, After 48h only a few Pax8-tdTomato-expressing cells were GFP-positive but the majority of GFP-expressing cells were Pax8-tdTomato-negative (Fig. 2C, top half), demonstrating that infection of epithelial cells by MuLE viruses is rare.

We nonetheless reasoned that it might be possible to transform epithelial cells and potentially create an *ex vivo* engineered endometrial carcinoma model by infecting pEECs with an ecotropic MuLE virus expressing sh*Cdkn2a* plus *H-Ras*^{G12V}, taking advantage of the fact that largely mutually exclusive genetic alterations in *KRAS*, *HRAS*, *NRAS*, *BRAF* and *NF1* occur in about 32% of human endometrial carcinomas (www.cbioportal.org) and loss of function of *Cdkn2a* in mouse cells allows escape from Ras-induced senescence and transformation (43,44). Infection of the above-described genetically labelled pEEC cultures with the sh*Cdkn2a* and *H-Ras*^{G12V}-expressing lentivirus lead to the emergence of a population of apparently transformed cells that proliferated rapidly, could be sequentially passaged and formed stream-like structures in culture, however these cells were negative for tdTomato expression (Fig. 2C, lower half), indicating that they are not epithelial in origin. It is known that endometrial epithelial cell cultures can be contaminated by small numbers of endometrial stromal cells and indeed, when these transformed cells were injected subcutaneously into SCID/beige mice they formed tumours with a sarcoma-like morphology. Histologically, the tumours stained negatively for CD31, DESMIN, MYOD1, MYOGENIN, α-SMA and positively for VIMENTIN (Fig. 2D). This molecular phenotype is consistent with a diagnosis of undifferentiated sarcoma of likely mesenchymal cell origin. We conclude

that while endometrial epithelial cells can be infected by MuLE viruses, albeit at low efficiency, transformation of other stromal cell types present in these cultures occurs efficiently by *H-Ras*^{G12V} overexpression and *Cdkn2a* knockdown.

Stromal cells are preferentially transduced by lentiviruses following *in vivo* injection

To study the efficacy of ecotropic lentiviral infection *in vivo,* we first injected mice with GFP-, tdTomato- or Luciferase-expressing ecotropic lentiviruses, but we were unable to detect the expression of any of these markers by immunofluorescence staining (data not shown). However, using R26-lox-STOP-lox-tdTomato mice (41) as a reporter system (Fig. 3A) to test cellular infection, we discovered that the injection of ecotropic lentiviruses expressing Cre into the uterus of cycling (n=3), pseudopregnant (n=1) and ovarectomized (n=1) mice induced tdTomato expression in cells in the uterine stroma but did not lead to infection of endometrial epithelial cells (Fig. 3B, first column). Since it has been shown that adenoviruses can infect stromal and epithelial cells *in vivo* (11,34,45)*,* we injected adenovirus expressing Cre into the uterus of cycling (n=2), pseudopregnant (n=1) and ovarectomized (n=1) mice as a positive control (Fig. 3B, second column). In ovarectomized mice we were indeed able to detect tdTomato-positive epithelial cells but again there were many more positive stromal cells. We conclude that lentiviral and adenoviral intra-uterine injections strongly favour infection of stromal cells rather than epithelial cells, even in mice in which endometrial cycling was hormonally blocked (ovarectomy, pseudopregnancy) to prevent the loss of potentially infected epithelial cells.

Intrauterine injection of MuLE viruses expressing shCdkn2a plus H-Ras^{G12V} causes sarcoma development

Given the data obtained above, we reasoned that it might be possible to generate a mouse model of uterine sarcomas. Since MuLE lentiviruses can be used to model undifferentiated pleomorphic sarcomas *in vivo* (36), a tumour of mesenchymal origin (34), concentrated ecotropic MuLE lentiviruses which cause either *Cdkn2a* silencing together with activation of *H-Ras* or *Pten* or *Trp53* silencing alone, were injected into the uterus of 4-week-old SCID/beige mice (Fig. 4A). Additionally, the MuLE vectors

carried a coding sequence for firefly luciferase in order to label infected cells and to trace tumour development over time. The injection of the sh*Cdkn2a* plus *H-Ras*^{G12V} expressing ecotropic MuLE lentivirus into the uterus of SCID/beige mice caused the development of high-grade endometrial stromal sarcomas (n=5) with 80% penetrance after less than two months (median overall survival 49 days) (Figure 4B, D, E). This process was monitored by an increase in luciferase expression over time (Figure 4C). Histologically, the tumours were highly cellular showing a diffuse growth pattern, delicate slit-like capillary network (Figure 4J, and extensive myometrial invasion. In addition, tumours were rapidly growing (Ki-67 proliferation fraction 10-50%, Supplementary Figure 1A) with high-grade cytologic atypia, resembling endometrial stromal sarcoma. Besides hemosiderin deposits (Supplementary Figure 1B), they stained positive for CYCLIN D1 (Figure 4G), diffusely positive for CD10 (Figure 4H) and VIMENTIN (Figure 4I) and negative for CD31 (Figure 4J), DESMIN (Figure 4K), MYOGENIN (Figure 4L) and α-SMA (Figure 4M). These cellular and molecular features are consistent with a diagnosis of high grade endometrial stromal sarcoma.

The injection of this virus into immunocompetent mice, namely C57BL/6 mice, did not cause tumour development within 6 months after injection (data not shown). None of the other injected vectors expressing knockdown constructs for two of the most commonly altered genes in endometrial carcinoma patients, namely *Pten* or *Trp53,* were sufficient to cause any large increases in luciferase signal over time and to induce tumour formation within 6 months after injection (Fig. 4B). The development of endometrial stromal sarcoma by the injection of sh*Cdkn2a* plus *H-Ras*^{G12V} expressing ecotropic lentiviruses serves as a technical proof-of-principle that this approach can be used to model uterine sarcomas.

DISCUSSION

In this study we employed a mouse genetic approach using the MuLE lentiviral gene regulatory system, (36)) to functionally test the ability of ecotropic lentiviruses to model epithelial and mesenchymal uterine malignancies *ex vivo* and *in vivo*. We discovered that MuLE lentiviruses efficiently infect uterine stromal cells but not endometrial epithelial cells when injected into the uterus of cycling, pseudopregnant or ovarectomized mice. Consistent with this cellular infection spectrum, we show that intra-uterine injection of ecotropic MuLE viruses expressing oncogenic *H-Ras*^{G12V} together with knockdown of *Cdkn2a* induce high-grade endometrial stromal sarcomas, establishing this approach as an efficient method of generating autochthonous mouse models of uterine sarcomas.

A major problem in endometrial cell culture-based studies are contaminating stromal cells, which are a byproduct of the isolation. Long term cultures have been proven to be particularly challenging because of contaminating stromal cells overgrowing the culture. While epithelial cells loose their proliferative capacity during ongoing cultivation on plastic, stromal cells are more easily cultured long term (40). Furthermore, the propagation of epithelial cells is limited by the early onset of senescence. Others have observed that a Rho kinase inhibitor (ROCK), in combination with fibroblast feeder cells, induces epithelial cells from many tissues to proliferate indefinitely *ex vivo*, without the need to immortalise them (46,47). The culture of our pEECs with conditional reprogramming by feeder cells and ROCK inhibitor did not work for us. Despite some minor growth in culture, our cells displayed strong degenerative changes, such as giant intra-nuclear vacuoles, and did not survive the first passage in culture (data not shown).

Since it has not been possible to expand and passage pEECs which retain lineage commitment and normal growth, we tried to generate immortalised cell lines through *Trp53* tumour suppressor inactivation or *H-Ras* oncogene activation with the use of ecotropic lentiviruses. This approach did not work, possibly due to the fact that ecotropic lentiviruses seem to efficiently infect contaminating stromal cells. Others have observed that mucin, a glycoconjugate, inhibits the entry of lenti- and adenoviruses into epithelial cells. They identified that the inbition of transduction could be improved by pretreating either the cells or the virus with the glycolyse hydrolase

neuraminidase before infection (48,49). It would be worth testing in the future if the pretreatment of either ecotropic lentiviruses or pEECs with neuraminidase increases epithelial cell transduction.

In vivo studies have shown that, when using adenoviral vectors, the infection efficiency entirely relied on the stage of the estrous cycle at the time of injection with best results occuring during late metestrous and diestrous stages of the estrous cycle (45). This is believed to be due to differences in mucus content and composition during the different phases of the estrous cycle, which influence virus entry. Furthermore, the estrous cycle in mice is only 4-6 days in length and continuous unless disrupted by pregnancy. During the estrous cycle apoptosis is increased tenfold in the luminar epithelium compared to other cell compartments (50). Even if epithelial cells were initially infected, the high rates of apoptosis may lead to the rapid loss of these cells during the cycle. By the use of R26-lox-STOP-lox-tdTomato mice (41) as a reporter system to test cellular infection, we discovered that the injection of ecotropic lentiviruses expressing Cre into the uterus of cycling, pseudopregnant and ovarectomized mice induced tdTomato expression in various cell types except epithelial cells regardless of stage of the estrous cycle and the viral dose. In rodent cells a cationic amino acid transporter, termed CAT1, serves as the receptor for the envelope glycoprotein gp70 of ecotropic MMLV (51). In the endometrium CAT1 is expressed on the basal surface of the epithelial cells (52). Limited accessibility of CAT1 could be one possible reason why we were not able to infect epithelial cells *in vivo*. Additionally, we injected Cre-liposome complexes into the uterine lumen of pseudopregnant and ovarectomized mice but also with this approach we detected reporter gene activity only in stromal cells (data not shown).

Only when we injected Cre-expressing adenoviruses in ovarectomized mice, we were able to see not only tdTomato-positive stromal cells but also very few positive epithelial cells. This confirmed the earlier observations that adenoviruses infect only few epithlial cells and many more stromal cells when injected in the uterus of lacZ reporter mice (11). The injection of an adenovirus expressing Cre into the uterus of *Pten*$^{fl/fl}$ mice lead to *Pten* deletion in both stromal and epithelial cells, however this treatment caused endometrial carcinoma development, but none of the injected uteri exhibited stromal tumours. This indicates that the deletion of *Pten* in stromal cells does not offer an

advantage to the cells and therefore is not selected for (11). Our studies in which injected ecotropic MuLE lentiviruses expressing sh*Pten* failed to induce stromal tumours when injected into the uterine lumen of SCID/beige mice are consistent with these data.

As proof-of-principle that ecotropic MuLE lentiviruses can be used to model stromal cell-derived tumours, discovered that the intrauterine injection of an ecotropic lentivirus expressing oncogenic *H-Ras* together with the knockdown of *Cdkn2a* was sufficient to initiate endometrial sarcoma formation in SCID/beige mice. This model is analagous to a similar aggressive uterine soft tissue sarcoma model that was previously generated through a Cre-loxP germline strategy. In this model, uterus-specific deletion of *Trp53* was combined with expression of a constitutively active form of *K-Ras* (34). The advantage of the MuLE system is that it is not dependent on the prior generation of germline-modified, Cre-regulatable mice and is therefore better suited to higher throughput testing of the pathological effects of different combination genetic alterations.

In summary, we discovered that ecotropic MuLE lentiviruses are able to inefficiently transduce epithelial cells *ex vivo* although infection strongly favor stromal cells. The injection of ecotropic lentiviruses into the uterus of mice infected stromal cells but not epithelial cells regardless of stage of the estrous cycle. The MuLE system appears to be suited for use in modelling endometrial sarcomas but not carcinomas.

REFERENCES

1. Bansal N, Yendluri V, Wenham RM. The molecular biology of endometrial cancers and the implications for pathogenesis, classification, and targeted therapies. Cancer Control. 2009;16(1):8–13.

2. Dedes KJ, Wetterskog D, Ashworth A, Kaye SB, Reis-Filho JS. Emerging therapeutic targets in endometrial cancer. Nature Reviews Clinical Oncology. Nature Publishing Group; 2011 Jan 11;8(5):261–71.

3. Reed N. A review of treatment of uterine leiomyosarcomas. Curr Oncol Rep. 2013 Dec;15(6):581–7.

4. Di Cristofano A, Ellenson LH. Endometrial Carcinoma. Annual Review of Pathology: Mechanisms of Disease. Annual Reviews; 2007 Feb;2(1):57–85.

5. Lax SF, Kurman RJ. A dualistic model for endometrial carcinogenesis based on immunohistochemical and molecular genetic analyses. Verh Dtsch Ges Pathol. 1997;81:228–32.

6. Daikoku T, Hirota Y, Tranguch S, Joshi AR, DeMayo FJ, Lydon JP, et al. Conditional loss of uterine Pten unfailingly and rapidly induces endometrial cancer in mice. Cancer Res. American Association for Cancer Research; 2008 Jul 15;68(14):5619–27.

7. Levine RL, Cargile CB, Blazes MS, van Rees B, Kurman RJ, Ellenson LH. PTEN mutations and microsatellite instability in complex atypical hyperplasia, a precursor lesion to uterine endometrioid carcinoma. Cancer Res. 1998 Aug 1;58(15):3254–8.

8. Oda K. High Frequency of Coexistent Mutations of PIK3CA and PTEN Genes in Endometrial Carcinoma. Cancer Res. American Association for Cancer Research; 2005 Dec 1;65(23):10669–73.

9. Bell D, O'Hara. The genomics and genetics of endometrial cancer. Advances in Genomics and Genetics. Dove Press; 2012 Mar;Volume 2:33–47.

10. Velasco A, Bussaglia E, Pallares J, Dolcet X, Llobet D, ENCINAS M, et al. PIK3CA gene mutations in endometrial carcinoma. Correlation with PTEN and K-RAS alterations☆. Human Pathology. 2006 Nov;37(11):1465–72.

11. Joshi A, Ellenson LH. Adenovirus mediated homozygous endometrial epithelial Pten deletion results in aggressive endometrial carcinoma. Exp Cell Res. 2011 Jul 1;317(11):1580–9.

12. Kim TH, Wang J, Lee KY, Franco HL, Broaddus RR, Lydon JP, et al. The Synergistic Effect of Conditional Pten Loss and Oncogenic K-ras Mutation on Endometrial Cancer Development Occurs via Decreased Progesterone Receptor Action. J Oncol. Hindawi Publishing Corporation; 2010;2010(1):139087–9.

13. Prat J, Gallardo A, Cuatrecasas M, Catasús L. Endometrial carcinoma: pathology and genetics. Pathology. Elsevier; 2007 Feb;39(1):72–87.

14. An H-J, Logani S, Isacson C, Ellenson LH. Molecular characterization of uterine clear cell carcinoma. Mod Pathol. Nature Publishing Group; 2004 Feb 20;17(5):530–7.

15. Fadare O, Liang SX, Cagnur Ulukus E, Chambers SK, Zheng W. Precursors of Endometrial Clear Cell Carcinoma. The American Journal of Surgical Pathology. 2006 Dec;30(12):1519–30.

16. Zhang X, Liang SX, Jia L, Chen N, Fadare O, Schwartz PE, et al. Molecular Identification of “Latent Precancers” for Endometrial Serous Carcinoma in Benign-Appearing Endometrium. The American Journal of Pathology. 2009 Jun;174(6):2000–6.

17. Kuhn E, Wu RC, Guan B, Wu G, Zhang J, Wang Y, et al. Identification of Molecular Pathway Aberrations in Uterine Serous Carcinoma by Genome-wide Analyses. J Natl Cancer Inst. Oxford University Press; 2012 Oct 2;104(19):1503–13.

18. Le Gallo M, O'Hara AJ, Rudd ML, Urick ME, Hansen NF, O'Neil NJ, et al. Exome sequencing of serous endometrial tumors identifies recurrent somatic mutations in chromatin-remodeling and ubiquitin ligase complex genes. Nat Genet. Nature Research; 2012 Oct 28;44(12):1310–5.

19. Cancer Genome Atlas Research Network, Kandoth C, Schultz N, Cherniack AD, Akbani R, Liu Y, et al. Integrated genomic characterization of endometrial carcinoma. Nature. Nature Research; 2013 May 2;497(7447):67–73.

20. Wild PJ, Ikenberg K, Fuchs TJ, Rechsteiner M, Georgiev S, Fankhauser N, et al. p53 suppresses type II endometrial carcinomas in mice and governs endometrial tumour aggressiveness in humans. EMBO Molecular Medicine. EMBO Press; 2012 Aug;4(8):808–24.

21. Tropé CG, Abeler VM, Kristensen GB. Diagnosis and treatment of sarcoma of the uterus. A review. Acta Oncologica. Taylor & Francis; 2012 Jul 15;51(6):694–705.

22. Prat J, Mbatani '. Uterine sarcomas. Int J Gynaecol Obstet. 2015 Oct;131 Suppl 2:S105–10.

23. Bertsch E, Qiang W, Zhang Q, Espona-Fiedler M, Druschitz S, Liu Y, et al. MED12 and HMGA2 mutations: two independent genetic events in uterine leiomyoma and leiomyosarcoma. Mod Pathol. Nature Publishing Group; 2014 Aug;27(8):1144–53.

24. Ylisaukko-oja SK, Kiuru M, Lehtonen HJ, Lehtonen R, Pukkala E, Arola J, et al. Analysis of fumarate hydratase mutations in a population-based series of early onset uterine leiomyosarcoma patients. International Journal of Cancer. Wiley Subscription Services, Inc., A Wiley Company; 2006;119(2):283–7.

25. Hu J, Rao UNM, Jasani S, Khanna V, Yaw K, Surti U. Loss of DNA copy number of 10q is associated with aggressive behavior of leiomyosarcomas: a comparative genomic hybridization study. Cancer Genetics and Cytogenetics. 2005 Aug;161(1):20–7.

26. Hernando E, Charytonowicz E, Dudas ME, Menendez S, Matushansky I, Mills J, et al. The AKT-mTOR pathway plays a critical role in the development of leiomyosarcomas. Nature Medicine. 2007 May 13;13(6):748–53.

27. Koontz JI, Soreng AL, Nucci M, Kuo FC, Pauwels P, van Den Berghe H, et al. Frequent fusion of the JAZF1 and JJAZ1 genes in endometrial stromal tumors. Proceedings of the National Academy of Sciences. National Acad Sciences; 2001 May 22;98(11):6348–53.

28. Micci F. Cytogenetic and molecular genetic analyses of endometrial stromal sarcoma: nonrandom involvement of chromosome arms 6p and 7p and confirmation of JAZF1/JJAZ1 gene fusion in t(7;17). Cancer Genetics and Cytogenetics. 2003 Jul 15;144(2):119–24.

29. Nucci MR, Harburger D, Koontz J, Cin PD, Sklar J. Molecular Analysis of the JAZF1-JJAZ1 Gene Fusion by RT-PCR and Fluorescence In Situ Hybridization in Endometrial Stromal Neoplasms. The American Journal of Surgical Pathology. 2007 Jan 1;31(1):65–70.

30. Oliva E, de Leval L, Soslow RA, Herens C. High Frequency of JAZF1-JJAZ1 Gene Fusion in Endometrial Stromal Tumors With Smooth Muscle Differentiation by Interphase FISH Detection. The American Journal of Surgical Pathology. 2007 Aug 1;31(8):1277–84.

31. Chiang S, Ali R, Melnyk N, McAlpine JN, Huntsman DG, Gilks CB, et al. Frequency of Known Gene Rearrangements in Endometrial Stromal Tumors. The American Journal of Surgical Pathology. 2011 Sep 1;35(9):1364–72.

32. Ríos I, Rovirosa A, Morales J, Gonzalez-Farre B, Arenas M, Ordi J, et al. Undifferentiated uterine sarcoma: a rare, not well known and aggressive disease: report of 13 cases. Arch Gynecol Obstet. Springer Berlin Heidelberg; 2014 Nov;290(5):993–7.

33. Tanner EJ, Garg K, Leitao MM Jr., Soslow RA, Hensley ML. High grade undifferentiated uterine sarcoma: Surgery, treatment, and survival outcomes. Gynecologic Oncology. 2012 Oct;127(1):27–31.

34. Kirsch DG, Dinulescu DM, Miller JB, Grimm J, Santiago PM, Young NP, et al. A spatially and temporally restricted mouse model of soft tissue sarcoma. Nature Medicine. Nature Publishing Group; 2007 Aug 1;13(8):992–7.

35. Mito JK, Riedel RF, Dodd L, Lahat G, Lazar AJ, Dodd RD, et al. Cross Species Genomic Analysis Identifies a Mouse Model as Undifferentiated Pleomorphic Sarcoma/Malignant Fibrous Histiocytoma. Aziz SA, editor. PLoS ONE. Public Library of Science; 2009 Nov 30;4(11):e8075.

36. Albers J, Danzer C, Rechsteiner M, Lehmann H, Brandt LP, Hejhal T, et al. A versatile modular vector system for rapid combinatorial mammalian genetics. J Clin Invest. American Society for Clinical Investigation; 2015 Apr;125(4):1603–19.

37. Traykova-Brauch M, Schönig K, Greiner O, Miloud T, Jauch A, Bode M, et al. An efficient and versatile system for acute and chronic modulation of renal tubular function in transgenic mice. Nature Medicine. 2008 Sep;14(9):979–84.

38. Grant KS, Wira CR. Effect of mouse uterine stromal cells on epithelial cell transepithelial resistance (TER) and TNFalpha and TGFbeta release in culture. Biol Reprod. Society for the Study of Reproduction; 2003 Sep;69(3):1091–8.

39. Eritja N, Llobet D, Domingo M, Santacana M, Yeramian A, Matias-Guiu X, et al. A novel three-dimensional culture system of polarized epithelial cells to study endometrial carcinogenesis. The American Journal of Pathology. 2010 Jun;176(6):2722–31.

40. Kyo S, Nakamura M, Kiyono T, Maida Y, Kanaya T, Tanaka M, et al. Successful immortalization of endometrial glandular cells with normal structural and functional characteristics. The American Journal of Pathology. Elsevier; 2003 Dec;163(6):2259–69.

41. Madisen L, Zwingman TA, Sunkin SM, Oh SW, Zariwala HA, Gu H, et al. A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. Nat Neurosci. 2010 Jan;13(1):133–40.

42. Mittag J, Winterhager E, Bauer K, Grümmer R. Congenital Hypothyroid Female Pax8-Deficient Mice Are Infertile Despite Thyroid Hormone Replacement Therapy. Endocrinology. Endocrine Society; 2011 Apr 9;148(2):719–25.

43. Kamijo T, Zindy F, Roussel MF, Quelle DE, Downing JR, Ashmun RA, et al. Tumor Suppression at the Mouse INK4a Locus Mediated by the Alternative Reading Frame Product p19 ARF. Cell. Elsevier; 1997 Nov;91(5):649–59.

44. Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell. 1997 Mar 7;88(5):593–602.

45. Beauparlant SL, Read PW, Di Cristofano A. In vivo adenovirus-mediated gene transduction into mouse endometrial glands: a novel tool to model endometrial cancer in the mouse. Gynecologic Oncology. 2004 Sep;94(3):713–8.

46. Liu X, Ory V, Chapman S, Yuan H, Albanese C, Kallakury B, et al. ROCK inhibitor and feeder cells induce the conditional reprogramming of epithelial cells. The American Journal of Pathology. Elsevier; 2012 Feb;180(2):599–607.

47. Narumiya S, Ishizaki T, Ufhata M. Use and properties of ROCK-specific inhibitor Y-27632. In: Regulators and Effectors of Small GTPases - Part D: Rho Family. Elsevier; 2000. pp. 273–84. (Methods in Enzymology; vol. 325).

48. Hines WC, Yaswen P, Bissell MJ. Modelling breast cancer requires identification and correction of a critical cell lineage-dependent transduction bias. Nat Commun. Nature Publishing Group; 2015 Apr 21;6:6927.

49. Arcasoy SM, Latoche J, Gondor M, Watkins SC, Henderson RA, Hughey R, et al. MUC1 and Other Sialoglycoconjugates Inhibit Adenovirus-mediated Gene Transfer to Epithelial Cells. American Journal of Respiratory Cell and Molecular Biology. American Thoracic SocietyNew York, NY; 1997 Oct;17(4):422–35.

50. Wood GA, Fata JE, Watson KLM, Khokha R. Circulating hormones and estrous stage predict cellular and stromal remodeling in murine uterus. Reproduction. Society for Reproduction and Fertility; 2007 May;133(5):1035–44.

51. Kim JW, Closs EI, Albritton LM, Cunningham JM. Transport of cationic amino acids by the mouse ecotropic retrovirus receptor. Nature. Nature Publishing Group; 1991 Aug 22;352(6337):725–8.

52. Proteinatlas SLC7A1 tissue expression. http://www.proteinatlas.org/ENSG00000139514-SLC7A1/tissue.

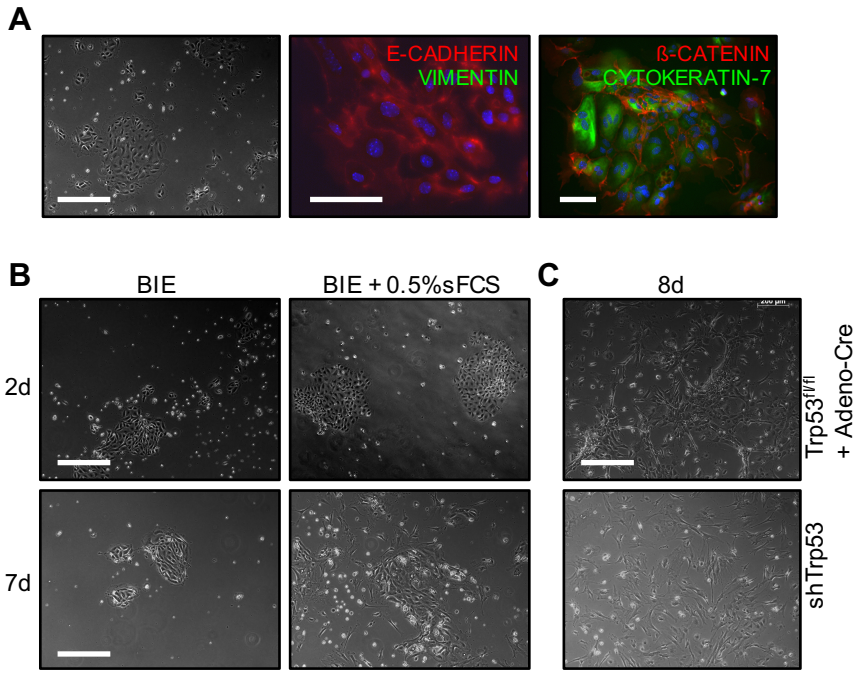


Figure 1. Establishment of a primary endometrial epithelial cell culturing system. (**A**) pEECs stain positively for the epithelial cell markers E-CADHERIN, ß-CATENIN and CYTOKERATIN-7 and negatively for the stromal cell marker VIMENTIN. Bright field image (10x) scale bar: 50 µm, low magnification (20x) scale bar: 100 µm and high magnification (63x) scale bar: 100 µm. (**B**) Supplementing BIE with 0.5% FCS promotes growth. BIE: basal medium supplemented with insulin, EGF, transferrin und sodium selenite. Scale bar: 50 µm. (**C**) Adeno-Cre-treated pEECs from *Trp53*$^{fl/fl}$ mice and pEECs infected with a lentivirus expressing a miR-30-based shRNA against *Trp53* were immortalised and could be expanded but lost epithelial morphology. Scale bar: 50 µm.

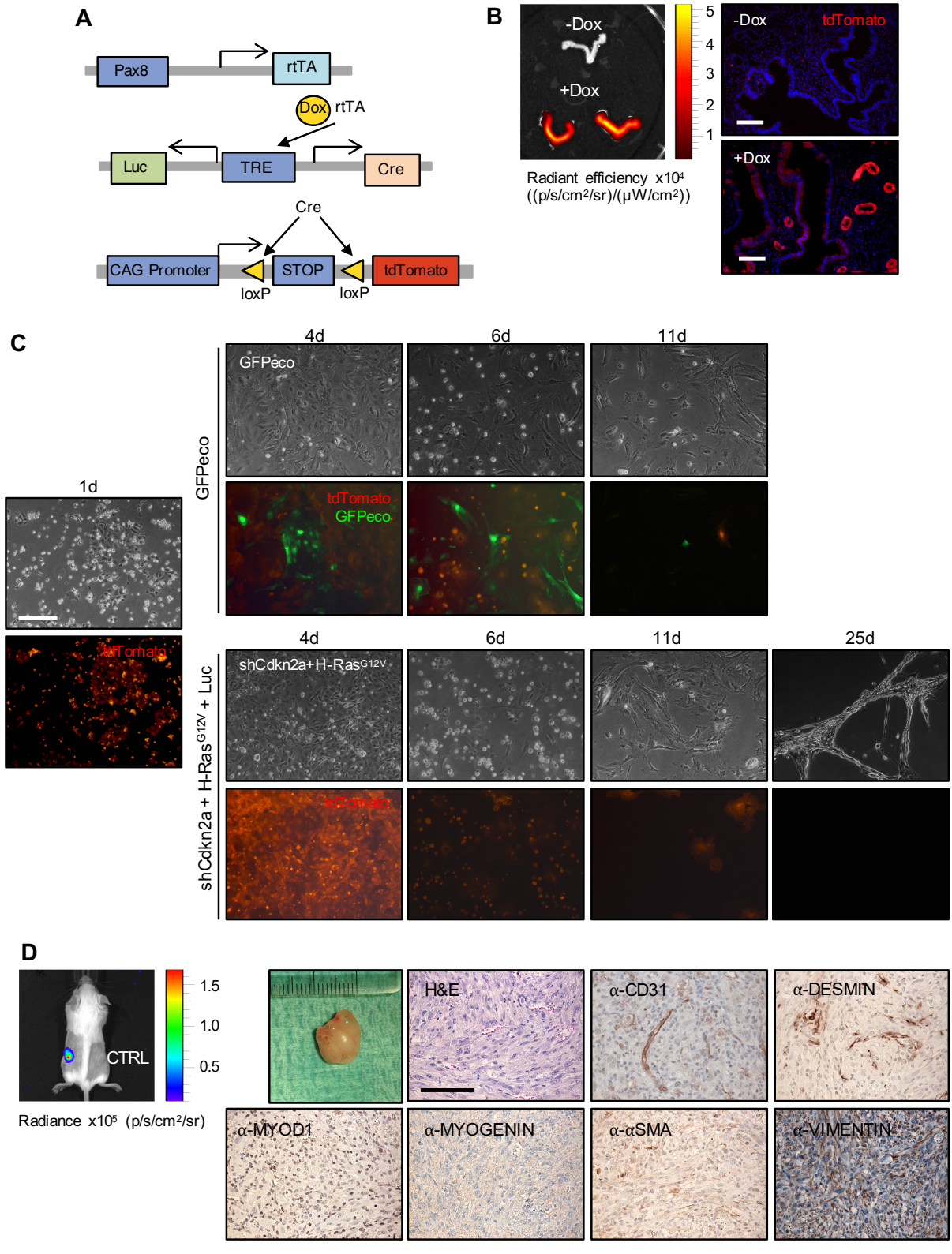

A
Pax8
rtTA
Dox
rtTA
Luc
TRE
Cre
Cre
CAG Promoter
STOP
tdTomato
loxP
loxP
B
-Dox
+Dox
5
4
3
2
1
Radiant efficiency x10⁴
((p/s/cm²/sr)/(μW/cm²))
-Dox
tdTomato
+Dox
C
4d
6d
11d
GFPeco
1d
tdTomato
GFPeco
tdTomato
GFPeco
4d
6d
11d
25d
shCdkn2a+H-RasG12V
tdTomato
shCdkn2a + H-RasG12V + Luc
D
CTRL
1.5
1.0
0.5
Radiance x10⁵ (p/s/cm²/sr)
H&E
α-CD31
α-DESMIN
α-MYOD1
α-MYOGENIN
α-αSMA
α-VIMENTIN

Figure 2. Lentiviral transduction of cultured uterine cells strongly favors stromal cells. (**A**) Schematic diagram of Pax8-rtTA-mediated doxycycline-controlled Cre-expression in Pax8-rtTA; LC1; ROSA26-lox-stop-lox tdTomato mice. (**B**) Doxycycline activates Cre-mediated recombination in glandular und luminar epithelial cells in the endometrium shown by whole organ imaging (left panel) and imaging of frozen histological sections (right panels). Scale bar: 100 µm. (**C**) Bright field and fluorescence images 1, 4, 6, 11 and 25 days after the isolation of pEECs from the uteri of doxycycline-treated Pax8-rtTA; LC1; ROSA26-lox-stop-lox tdTomato mice. pEECs were infected on day 1 with ecotropic lentiviruses expressing either GFP or shCdkn2a plus H-Ras^{G12V} and Luciferase. Scale bar: 50 µm. (**D**) Ability of shCdkn2a and H-Ras^{G12V}-transduced cells to form tumours within 47 days in allograft experiments (visualised using luciferase imaging in the left panel). Right panels show H&E and immunohistochemical stainings using the indicated antibodies. Low magnification scale bar: 1 cm and high magnification scale bar: 100 µm.

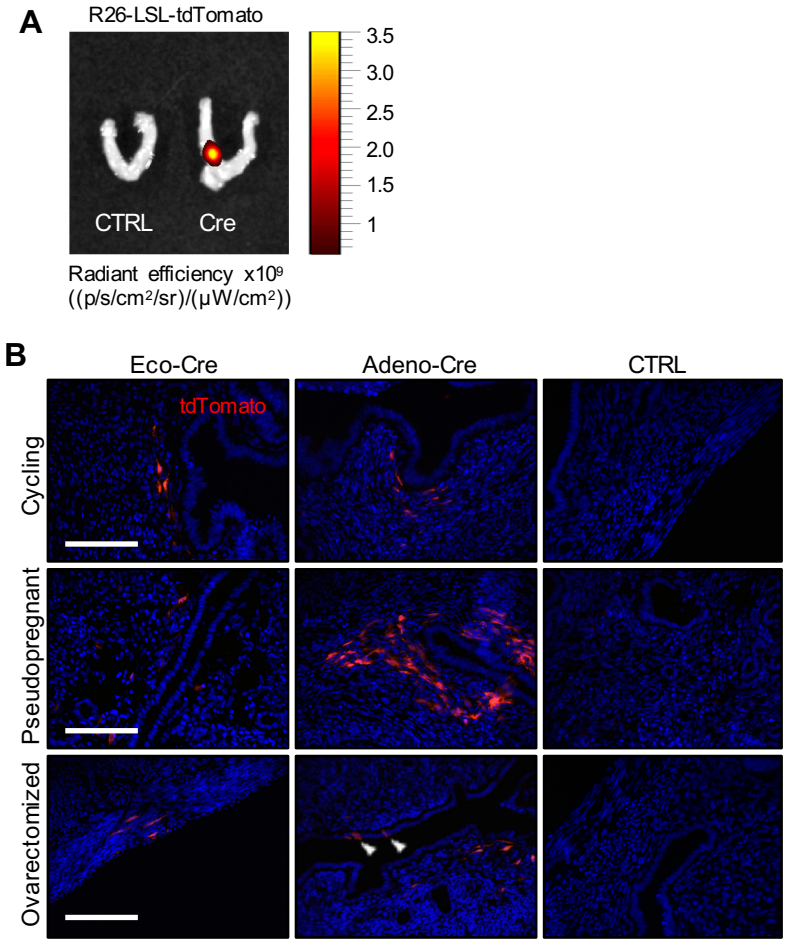


Figure 3. Stromal cells are preferentially transduced by lentiviruses following *in vivo* injection. (**A**) tdTomato fluorescence signal in the uterus following intrauterine injection of ecotropic virus expressing Cre. (**B**) Intrauterine injection of ROSA26-lox-STOP-lox-tdTomato mice with Cre-expressing lenti- and adenoviruses. Arrowheads indicate infected epithelial cells. Scale bar: 100 µm. CTRL indicates non-injected animals.

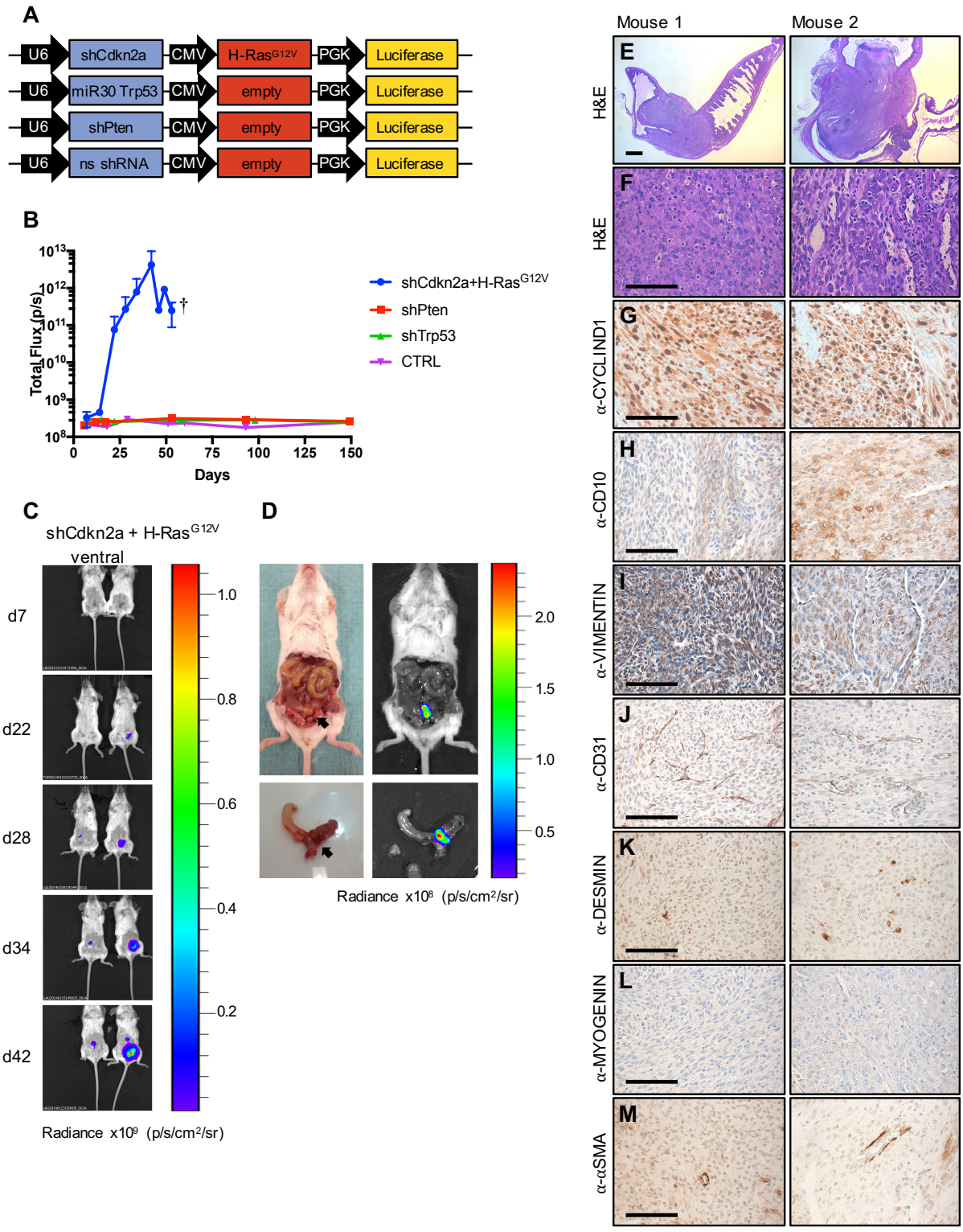
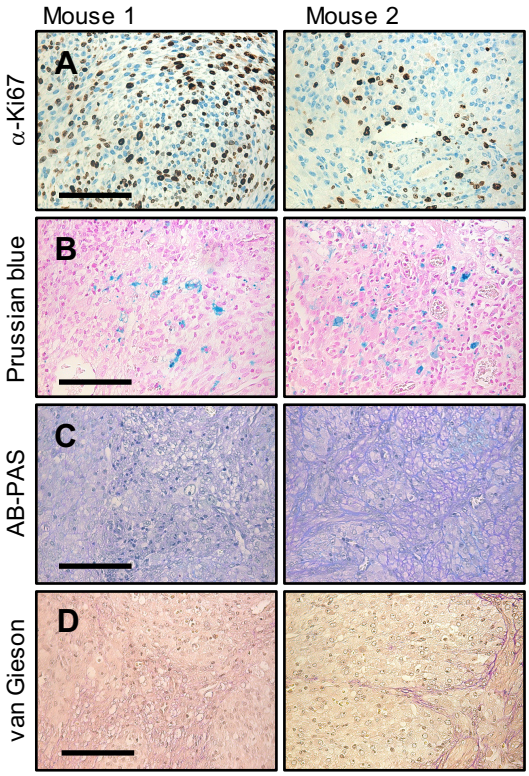

A
U6
shCdkn2a
CMV
H-Ras^{G12V}
PGK
Luciferase
U6
miR30 Trp53
CMV
empty
PGK
Luciferase
U6
shPten
CMV
empty
PGK
Luciferase
U6
ns shRNA
CMV
empty
PGK
Luciferase
B
Total Flux (p/s)
Days
shCdkn2a+H-Ras^{G12V}
shPten
shTrp53
CTRL
C
shCdkn2a + H-Ras^{G12V}
ventral
d7
d22
d28
d34
d42
Radiance x10^{9} (p/s/cm^{2}/sr)
D
Radiance x10^{8} (p/s/cm^{2}/sr)
Mouse 1
Mouse 2
H&E
H&E
α-CYCLIND1
α-CD10
α-VIMENTIN
α-CD31
α-DESMIN
α-MYOGENIN
α-αSMA

Figure 4. *H-Ras*^{G12V} expression plus knockdown of *Cdkn2a* causes high-grade endometrial stromal sarcomas. (**A**) Schematic of MuLE vectors simultaneously expressing a combination of shRNA against *Cdkn2a* plus expression of *H-Ras* or expressing shRNAs against *Trp53* or *Pten.* All vectors also expressed Luciferase. (**B**) Quantification (mean ± SD) of luciferase signal over time. † Sacrifice of all mice in this group by this time point. (**C**) Bioluminescence imaging in two mice 7, 22, 28, 34 and 42 days after the injection of MuLE lentiviruses expressing shRNA against *Cdkn2a* together with *H-Ras*^{G12V} into the uterus of 6-8-week-old SCID/beige mice. Injected mice developed tumours (n=5) with 80% penetrance. Median overall survival was 49 days. (**D**) Bioluminescence imaging and photographs of tumour-bearing uterus. (**E**-**M**) H&E and immunohistochemical stainings of tumours from two different mice using the indicated antibodies. Low magnification scale bar: 1000 µm and high magnification scale bar: 100 µm.



Supplementary Figure 1. Generation of high-grade endometrial stromal sarcomas. (**A**) Uterine tumours show increased proliferation (Ki67 immunohistochemistry), (**B**) hemosiderin deposition (Prussian blue staining), (**C**) absence of mucin inclusions (Alcian Blue PAS staining) and (**D**) smooth muscle cells in yellow and collagen in red (van Gieson's staining). Scale bar: 100 µm.

2.2 Mouse genetic background dictates whether *H-Ras*^{G12V} expression plus *Cdkn2a* knockdown causes angiosarcoma or undifferentiated pleomorphic sarcoma

Manuscript in preparation

Mouse genetic background dictates whether *H-Ras^{G12V}* expression plus *Cdkn2a* knockdown causes angiosarcoma or undifferentiated pleomorphic sarcoma

Laura P. Brandt[1,2], Joachim Albers[1], Tomas Hejhal[1], Svende Pfundstein[1,3], Ana Filipa Gonçalves[1], Peter J. Wild[4], Ian J. Frew[1,2,5,6].

[1] Institute of Physiology, University of Zurich, Zurich, Switzerland
[2] Zurich Center for Integrative Human Physiology, University of Zurich, Zurich, Switzerland
[3] Zurich Integrative Rodent Physiology, University of Zurich, Zurich, Switzerland
[4] Department of Pathology and Molecular Pathology, University Hospital Zurich, Zurich, Switzerland
[5] BIOSS Center for Biological Signalling Studies, University of Freiburg, Freiburg, Germany
[6] Center for Translational Cell Research, Clinic of Internal Medicine I, Medical Center - University of Freiburg, Freiburg, Germany

Address for correspondence:
Ian Frew
Center for Translational Cell Research
Medical Center - University of Freiburg
Breisacher Straße 115
79106 Freiburg
Germany

Phone: +49 (0) 761 270 71831
Email: ian.frew@uniklinik-freiburg.de

ABSTRACT

Soft tissue sarcomas are rare mesenchymal tumours accounting for 1% of adult malignancies and are fatal in approximately one third of patients. Two of the most aggressive and lethal forms of soft tissue sarcomas are angiosarcomas and undifferentiated pleomorphic sarcomas (UPS). To examine sarcoma-relevant molecular pathways, we employed a lentiviral gene regulatory system to attempt to generate *in vivo* models that reflect common molecular alterations of human angiosarcoma and UPS. Mice were systemically injected with MuLE lentiviruses expressing combinations of shRNA against *Cdkn2a*, *Trp53, Tsc2* and *Pten* with or without expression of *H-Ras, PIK3CA* and *c-Myc*. The systemic injection of an ecotropic lentivirus expressing oncogenic *H-Ras* together with the knockdown of *Cdkn2a* or *Trp53* was sufficient to initiate angiosarcoma and/or UPS development, providing a flexible system to generate autochthonous mouse models of these diseases. Unexpectedly, different mouse strains developed different types of sarcoma in response to identical genetic drivers, implicating genetic background as a major contributor to the genesis and spectrum of sarcomas.

INTRODUCTION

Soft tissue sarcomas are rare mesenchymal malignancies which account for approximately 1% of all cancers. The WHO has defined over 100 different soft tissue sarcoma subtypes named after the tissue that they most closely resemble (1). Based on molecular characteristics, soft tissue sarcomas can be divided in two categories: low-grade sarcomas with simple karyotypes, such as chromosomal translocations, and high-grade sarcomas with more complex genetic profiles, including *TP53* mutation, *CDKN2A* deletion and *MDM2* amplification (2-4). Genetically complex sarcomas are typically clinically more aggressive than karyotypically simple sarcomas.

Undifferentiated pleomorphic sarcomas (UPS) account for approximately 5% of adult soft tissue sarcomas and represent one of the most common types of high-grade soft tissue sarcoma. Standard treatment options are surgical resection, radiotherapy, and chemotherapy, which only provide modest increases in the overall survial of these patients. Novel targeted treatments are needed. It is not clear whether UPS represents a group of de-differentiated sarcomas which share a common morphology but originated from different cell types or if all UPS tumours arise from a common cell of origin (5). The genetic alterations responsible for the development of UPS are also incompletely understood. *TP53* alterations have been identified in 17% of human UPS (2) and *CDKN2A* loss seems to be an alternative to *TP53* deletion (3). Despite the fact that p53 pathway inactivation is a common event in high-grade sarcomas, additional mutations are required for sarcomagenesis. *HRAS* and *KRAS* mutations have been identified in up to 50% of the human UPS tumours (6,7). Mouse studies have confirmed that the cooperation of oncogenic *Kras* and *Trp53* or *Cdkn2a* deficiency resulted in the development of undifferentiated pleomorphic sarcomas in different tissues (8-11).

A much less frequent but equally clinically aggressive subtype of high-grade soft tissue sarcomas are angiosarcomas. These tumours represent rare malignancies of endothelial differentiation that account for approximately 1% of all soft tissue sarcomas. Angiosarcomas show a wide anatomic distribution and arise spontaneously or secondarily to radiation, toxic chemicals (e.g. vinyl chloride) or chronic lymphoedema (Stewart-Treves syndrome). Treatment options are limited and the prognosis is poor (12). Genetic mutations and amplifications of *VEGF*, *MDM2*, *TP53*,

CDKN2A, *KRAS* and *MYC* have been described in angiosarcoma patients (13-16). *MYC* gene amplifications are commonly found in a subset of radiation-induced angiosarcomas (17). A recent publication reported that the majority of genetic alterations were found in the p53 and MAPK pathways. *TP53* was mutated in 35% of the lesions and *CDKN2A* lost in 26%. 53% of angiosarcomas displayed MAPK pathway activation, and harboured genetic activating mutations in *KRAS*, *HRAS*, *NRAS*, *BRAF*, *MAPK1* or inactivating mutations in *NF1* and *PTPRB1* (18,19). Several *in vivo* mouse studies showed the involvement of loss of function of the p53 tumour suppressor in angiosarcoma development (20-22). In addition, the *in vivo* deletion of *Cdkn2a* in mice lead to the development of lesions which recapitulate human angiosarcoma, however, only 30% of the mice displayed angiosarcomas within 100 days (23). Furthermore, alterations in the PI3K/AKT/mTOR pathway have been identified in a small percentage of patients (18,24,25) and deletion of *Tsc1*, a tumour suppressor that negatively regulates the pathway, induced the formation of hemangiosarcomas in mice (26). Another report showed that the *in vivo* deletion of *Notch1* resulted in the development of hepatic angiosarcomas with a penetrance of 86% at 50 weeks after gene deletion (27), although genetic alterations in the Notch pathway, have not been reported in human angiosarcomas. Although these studies have been helpful in uncovering aspects of sarcomagenesis, there is limited understanding of the interactions between cooperating genetic alterations.

In this study we employed a mouse genetic approach using the MuLE lentiviral gene regulatory system, (9)) to functionally test the contributions of different candidate driver oncogenes and tumour suppressor genes to the formation of angiosarcoma and UPS. Different mouse strains were injected intraveneously with ecotropic MuLE lentiviruses expressing combinations of shRNA against *Cdkn2a*, *Trp53, Tsc2* and *Pten* with or without expression of *H-Ras, PIK3CA* and *c-Myc*. Tumour development was monitored by *in vivo* imaging. We successfully generated new models of angiosarcoma and of UPS based on oncogenic *H-Ras* expression in combination with knockdown of *Cdkn2a* or *Trp53*. Unexpectedly, different mouse strains developed different types of sarcoma in response to identical genetic drivers.

MATERIALS AND METHODS

Mice

SCID/beige mutant mice (C.B-17/CrHsd-Prkdc^{Scid}Lyst^{bg-J}) and C57BL/6JRccHsd mice were obtained from Envigo. 129S2/SvPasOrlRj mice were obtained from Janvier Labs and Fox Chase CB17^{TM} mice (C.BKa-Igh^{b}/IcrCrl) were obtained from Charles River Laboratories. All mouse experiments were approved by the Veterinary Office of the Canton of Zurich under the licence 137/2013.

Generation of MuLE vectors

The majority of the MuLE Entry and Destination vectors used in this study were previously described (9) and were recombined using MultiSite Gateway LR 2-fragment recombinations to generate the final viral constructs. New MuLE Entry vectors carrying 7SK promotor-driven expression of shRNA against *Tsc2* (Sigma, TRCN0000306244) and CMV promotor-driven expression of hemagglutinin (HA) tagged phosphoinositide 3-kinase H1047R (HA-PIK3CA H1047R, Addgene 12524, deposited by Dr. Jean Zhao) were generated. Ecotropic lentiviral vectors were produced by using calcium phosphate-mediated transfection of HEK293T cells and the viral preparation was concentrated as described previously (9).

In vivo tumour formation assays

10 ml/kg of concentrated ecotropic lentiviruses were injected through a 30G insulin syringe into the lateral tail vein of 4-6-week old mice. Noninvasive *in vivo* bioluminescence imaging was performed using the IVIS Spectrum (Perkin Elmer) together with the Living Image software (version 4.4). Mice were anaesthetized by using 2.5% isoflurane. During imaging, the isoflurane levels were reduced to 1.5%. All fluorescence measurements were performed in epi-fluorescence mode. For bioluminescence imaging, mice were injected subcutaneously with 150 mg/kg D-luciferin (Caliper, no. 122796) and imaged 15 min after injection.

Immunohistochemistry

Tumour-bearing organs were resected, fixed in 10% formalin, paraffin-embedded and cut in 5 µm thick sections. Immunohistochemical analysis was performed after antigen retrieval (5 min at 110°C in 0.1M citrate buffer pH6). The antibodies used in this study were anti-CD31 (1:200, ab28364, abcam), anti-DESMIN (1:100, D1033, Sigma-Aldrich), anti-MYOD1 (1:100, M3512, Dako), anti-MYOGENIN (1:500, M3559, Dako), anti-alpha-SMA (1:5000, ab5694, abcam), anti-VIMENTIN (1:500, D21H3, Cell Signaling) and anti-von Willebrand Factor (vWF) (1:1000, Dako).

RESULTS

Expression of oncogenic *H-Ras*^{G12V} plus knockdown of *Cdkn2a* causes angiosarcoma development in SCID/beige mice

To functionally test the contributions of different candidate driver oncogenes and tumour suppressor genes to the formation of angiosarcoma, we generated a panel of lentiviral vectors based on the MuLE system (Fig. 1A), to induce genetic alterations that reflect some of the most commonly found alterations of human angiosarcomas. 4-6-week-old SCID/beige mice were intravenously injected via the tail vein with concentrated ecotropic MuLE lentiviruses expressing combinations of shRNA or shRNA-miR30 against *Cdkn2a, Trp53, Tsc*2 and *Pten* with or without expression of oncogenic *H-Ras*^{G12V}, oncogenic *PIK3CA*^{H1047R} and *c-Myc*. Additionally, MuLE vectors carried an expression element for firefly luciferase in order to label infected cells and to trace tumour development *in vivo* over time. Western blotting and real time PCR assays of cultured cells infected with these vectors verified that they effectively induced the desired changes in gene expression (Supplementary Fig. S1).

The intravenous injection of an ecotropic lentivirus expressing oncogenic *H-Ras*^{G12V} together with knockdown of *Cdkn2a* (n=21 of 32 injected mice) or *Trp53* (n=2 of 4 injected mice) in SCID/beige mice induced increases in luciferase signals over 4-8 weeks (Fig. 1B). These signals were widely distributed in different organs throughout the body. One of three mice injected with a vector expressing only oncogenic *H-Ras*^{G12V} developed signals in the brain approximately 6 months after injection, showing that loss of *Cdkn2a / Trp53*-governed tumour suppressor pathways accelerate *H-Ras*-driven oncogenesis. None of the other viruses was sufficient to cause any large increases in luciferase signal within 6 months of injection, demonstrating that these combinations of genetic alterations are not oncogenic in this setting.

Dissections of mice revealed that the increased luciferase signals corresponded to the presence of bloody-appearing tumours in different organs (Fig. 1C and D). sh*Cdkn2a* plus *H-Ras*^{G12V}-injected mice developed tumours faster and with a higher penetrance (median overall survival = 37 days, 66% penetrance) than sh*Trp53* plus *H-Ras*^{G12V}-injected mice (median overall survival = 64 days , 50% penetrance). Therefore, we decided to focus on the sh*Cdkn2a* and *H-Ras*^{G12V} combination for all further

experiments. From 32 mice injected with sh*Cdkn2a* plus *H-Ras*^{G12V} MuLE vectors, 21 mice developed a total of 24 tumours in various tissues including testicle (n=9, 38%), brain (n=7, 30%), spleen (n=2, 8%), uterus (n=2, 8%), ovary (n=1, 4%), lung (n=1, 4%), colon (n=1, 4%) and eye (n=1, 4%). Histological analysis of these tumours revealed poorly demarcated malignant neoplasms with hemorrhage and irregular, anastomosing vascular channels. Endothelial lining showed multilayering and intraluminal tufting with nuclear atypia, hyperchromasia, enlargement and irregularity (Supplementary Fig. S2A, arrowheads). Mitotic activity was variable and back-to-back vascular channels appeared sieve-like. Atypical cells were either spindled, epitheloid or mixed. There was more solid growth in more poorly differentiated areas. Intraluminal erythrocytes were a common feature as well as large areas filled with red blood cells. Positive immunoreactivity to antibodies against the endothelial cell marker proteins CD31 and von Willebrand Factor (vWF) confirmed the endothelial differentiation of the tumour cells. The lesions showed variable staining for VIMENTIN, DESMIN and SMOOTH MUSCLE ACTIN (SMA), ranging from an absence of staining to some tumours showing strong positivity. None of the tumours stained positively for the skeletal muscle markers MYOD1 and MYOGENIN (Fig. 2A, Supplementary Fig. S2B). Tumours that arose in *Trp53* knockdown plus oncogenic *H-Ras*^{G12V} injected mice displayed an identical histological appearance and immunohistochemical staining profile to tumours in *Cdkn2a* knockdown plus oncogenic *H-Ras*^{G12V} injected mice (Fig. 2B). In summary, these histological and molecular features are consistent with a diagnosis of angiosarcoma. We conclude that we have developed a rapid autochthonous mouse model of angiosarcoma that is trackable via live animal imaging and that reflects the frequent genetic alterations that arise in human angiosarcoma tumours.

Different immunocompetent mouse strains display different types of soft tissue sarcomas in response to expression of oncogenic *H-Ras*^{G12V} plus loss of *Cdkn2a* function

To more accurately model the complexities of tumour development in humans it would be desirable to have tumour models that arise in immunocompetent mice. We therefore investigated whether similar tumours arose in response to intravenous injection of

shCdkn2a plus *H-Ras*^{G12V} MuLE vectors in the Fox Chase CB17, 129/Sv and C57BL/6 mouse strains.

Fox Chase CB17 mice carry the immunoglobulin heavy chain allele from C57BL/Ka mice on a BALB/c background. They serve as an ideal control for SCID/beige mice as they represent the identical genetic background but have a normal immune system. Within 4 weeks, 88% of Fox Chase CB17 mice (n=7) injected intraveneously with sh*Cdkn2a* plus *H-Ras*^{G12V} MuLE vectors developed angiosarcomas with comparable growth kinetics and histology to those which arise in SCID/beige mice (Supplementary Fig. S3A). The anatomic distribution of the tumours in Fox Chase CB17 mice was similar to the tumour distribution seen in SCID/beige mice and included brain (n=4, 50%), testicle (n=3, 38%) and spleen (n=1, 12%) (Supplementary Fig. S3B). The tumours presented as bloody lesions and stained positively for CD31 and vWF by immunohistochemistry. Like the tumours in SCID/beige mice, these tumours also showed variable staining for VIMENTIN, DESMIN and SMA as well as an absence of staining for MYOD1 and MYOGENIN (Supplementary Fig. S3C). These experiments demonstrate that a competent immune system does not affect tumour formation and provide a new autochthonous angiosarcoma model in an immunocompetent background.

To investigate sarcoma formation in mouse backgrounds that are more commonly utilised for biomedical research we next employed 129/Sv mice. Intraveneous injections of sh*Cdkn2a* plus *H-Ras*^{G12V} MuLE viruses in 129/Sv mice (n=8) caused a strong luciferase signal increase and the development of multiple tumours with 100% penetrance within 4 weeks (Fig. 3A and B, Supplementary Fig. S4A). Bloody-appearing tumours were observed in testicles of 75% of male mice (n=3) and in uteri (n=2) and ovaries (n=2) of 50% of female mice. 25% of mice carried lesions in the spleen (n=2), and 13% in lung (n=1) and brain (n=1), similar to results in SCID/beige mice. However, all female mice (n=4) additionally developed subcutaneous tumours (n=5) that were located in the head and neck region and close to the vagina. These tumours were solid and white in appearance (Fig. 3C, last column). Neither the sub-cutaneous location, nor this gross morphological appearance were ever seen in tumours in SCID/beige or Fox Chase CB17 mice. While all of the bloody-appearing tumours exhibited an identical histological appearance and pattern of immunoreactivity

similar to those arising in SCID/beige mice (Fig. 3C, Supplementary Fig. S4B), classifying them as angiosarcomas, the subcutaneous tumours exhibited a completely different histology and immunohistochemical staining profile. These tumours contained cells with rhabdoid features; i.e., large polygonal cells with gigantic bizarre nuclei (Supplementary Fig. S5A; arrowheads), abundant deeply eosinophilic cytoplasm in a tadpole- or racquet shape, and growing in a storiform pattern. Nuclei displayed high mitotic indices, irregular nuclear membranes, and eosinophilic cytoplasmic inclusions. These tumours showed necrotic regions, acute inflammatory responses and were highly invasive, infiltrating surrounding tissues including muscle and fat (Supplementary Fig. S5A, arrows). These tumours were immunoreactive for the common mesenchymal marker VIMENTIN, but did not stain for any lineage markers including CD31, vWF, DESMIN, SMA, MYOD1 or MYOGENIN (Fig. 3C, last column). We, therefore, concluded that these tumours are high-grade undifferentiated pleomorphic sarcomas (UPS). Thus, 129/Sv mice develop both angiosarcomas and UPS in response to the same oncogenic stimulus.

To further investigate the effect of genetic background on tumour formation, we injected C57BL/6 mice with sh*Cdkn2a* plus *H-Ras*^{G12V} MuLE lentiviruses. Within 4-8 weeks of injection both male and female C57BL/6 mice showed large increases in luciferase signal and developed subcutaneous lesions with 92% penetrance (Fig. 4A and B). Tumours (n=12) that developed in C57BL/6 mice were solid and white in appearance like those seen in female 129/Sv mice. They were subcutaneous and located either in the head and neck area, lower leg or at the junction of the tail and spine. These tumours exhbited an identical histological appearance to the UPS tumours that arose in 129/Sv mice (Supplementary Fig. S5B). These tumours similarly showed an absence of staining for all tested markers, except VIMENTIN (Fig. 4C). Based on these results we conclude that sh*Cdkn2a* plus *H-Ras*^{G12V} causes solely high-grade UPS in C57BL/6.

DISCUSSION

Major hurdles in studying sarcoma pathologies are the relative rarities of the human diseases and the absence for many sarcoma subtypes of good pre-clinical models. Here, we used the MuLE lentiviral gene regulatory system (9) to investigate the molecular genetics underlying the pathogenesis of two types of soft tissue sarcomas, namely angiosarcoma and UPS. The MuLE system allows the direct introduction of multiple genetic alterations in somatic cells *in vivo* by lentiviral injection. Bypassing germline transgenic approaches has benefits in terms of time and costs and offers flexibility in terms of the strains of mice that can be used for the experiments, allowing comparisons between different genetic backgrounds. Guided by the genetics of human angiosarcomas and UPS tumours, we functionally tested the contributions of the candidate sarcoma tumour suppressors *Cdkn2a*, *Trp53, Tsc2* and *Pten* and the candidate oncogenes *H-Ras, PIK3CA* and *c-Myc*. We discovered that the systemic injection of ecotropic lentiviruses expressing oncogenic *H-Ras* together with the knockdown of *Cdkn2a* or *Trp53* was sufficient to initiate angiosarcoma formation in multiple organs in SCID/beige mice. sh*Cdkn2a* plus H-Ras^{G12V} MuLE viruses also induced angiosarcoma formation in Fox Chase CB17 and 129/Sv mice, but surprisingly additionally caused UPS development in 129/Sv mice and only UPS development in C57/BL6 mice. These observations are consistent with the fact that RAS-MAPK and p53 pathway alterations are frequently found in high-grade soft tissue sarcomas, such as angiosarcomas and UPS (2,3,6,7,18). Our experimental approach therefore accurately models at least some of the genetic subsets of these human tumours.

Genetic cooperation between RAS pathway activation and loss of *Cdkn2a* tumour suppressor function has also been shown in other mouse models of sarcomas. Intramuscular injection of Adeno-Cre in the leg of *loxP-STOP-loxP-K-Ras*$^{G12D/+}$;*Cdkn2a*$^{fl/fl}$ mice caused UPS development (8,10) and we have previously shown that intramuscular injection of the same sh*Cdkn2a* plus H-Ras^{G12V} or sh*Trp53* plus H-Ras^{G12V} MuLE lentiviruses that were used in this study caused the developement of high-grade UPS (9). It is noteworthy that injection of these viruses into skeletal muscle in SCID-beige mice caused UPS (9) but intravenous injection caused angiosarcoma, whereas in C57/BL6 mice both modes of injection caused UPS formation (9). Thus, our data demonstrate that the same combination of genetic drivers

can cause different types of tumours based not only on the delivery method, likely due to infection of different cell types, but also based on the genetic background of the mouse strain. These observations highlight the fact that also in humans the genetic background of every individual may potentially influence the outcome of oncogenic mutations in terms of what type of sarcoma develops and further emphasise the need to consider tumours at the molecular level rather than at the level of histo-pathological appearance when thinking about the development and clinical application of new molecularly-targeted sarcoma therapies.

In summary, these new experimental models will facilitate future pre-clinical studies for establishing new therapeutic interventions for these aggressive malignancies. While it is somewhat surprising that the other tested candidate tumour suppressors and oncogenes were not sufficient to cause tumour formation, given their frequent mutational alteration in human angiosarcomas and UPS, the flexible nature of the MuLE system should also allow the testing of other candidate oncogenes, modifier genes or tumour suppressor genes that will likely continue to emerge from ongoing genomic studies of these rare tumours.

REFERENCES

1. Fletcher C. WHO classification of tumours of soft tissue and bone. Fourth Edition. 2013.

2. Barretina J, Taylor BS, Banerji S, Ramos AH, Lagos-Quintana M, Decarolis PL, et al. Subtype-specific genomic alterations define new targets for soft-tissue sarcoma therapy. Nat Genet. 2010 Aug;42(8):715–21.

3. Pérot G, Chibon F, Montero A, Lagarde P, de Thé H, Terrier P, et al. Constant p53 Pathway Inactivation in a Large Series of Soft Tissue Sarcomas with Complex Genetics. The American Journal of Pathology. 2010 Oct;177(4):2080–90.

4. Borden EC, Baker LH, Bell RS, Bramwell V, Demetri GD, Eisenberg BL, et al. Soft tissue sarcomas of adults: state of the translational science. Clin Cancer Res. American Association for Cancer Research; 2003 Jun;9(6):1941–56.

5. Matushansky I, Charytonowicz E, Mills J, Siddiqi S, Hricik T, Cordon-Cardo C. MFH classification: differentiating undifferentiated pleomorphic sarcoma in the 21st Century. Expert Rev Anticancer Ther. 2009 Aug;9(8):1135–44.

6. Yoo J, Robinson RA, Lee JY. H-ras and K-ras gene mutations in primary human soft tissue sarcoma: concomitant mutations of the ras genes. Mod Pathol. 1999 Aug;12(8):775–80.

7. Yoo J, Robinson RA. H-ras and K-ras mutations in soft tissue sarcoma. Cancer. John Wiley & Sons, Inc; 1999 Jul 1;86(1):58–63.

8. Kirsch DG, Dinulescu DM, Miller JB, Grimm J, Santiago PM, Young NP, et al. A spatially and temporally restricted mouse model of soft tissue sarcoma. Nature Medicine. Nature Publishing Group; 2007 Aug 1;13(8):992–7.

9. Albers J, Danzer C, Rechsteiner M, Lehmann H, Brandt LP, Hejhal T, et al. A versatile modular vector system for rapid combinatorial mammalian genetics. J Clin Invest. American Society for Clinical Investigation; 2015 Apr;125(4):1603–19.

10. Hettmer S, Liu J, Miller CM, Lindsay MC, Sparks CA, Guertin DA, et al. Sarcomas induced in discrete subsets of prospectively isolated skeletal muscle cells. Proc Natl Acad Sci USA. National Acad Sciences; 2011 Dec 13;108(50):20002–7.

11. Blum JM, Añó L, Li Z, Van Mater D, Bennett BD, Sachdeva M, et al. Distinct and Overlapping Sarcoma Subtypes Initiated from Muscle Stem and Progenitor Cells. Cell Reports. 2013 Nov;5(4):933–40.

12. Young RJ, Brown NJ, Reed MW, Hughes D, Woll PJ. Angiosarcoma. The Lancet Oncology. 2010 Oct;11(10):983–91.

13. Zietz C, Rössle M, Haas C, Sendelhofert A, Hirschmann A, Stürzl M, et al. MDM-2 Oncoprotein Overexpression, p53 Gene Mutation, and VEGF Up-Regulation in Angiosarcomas. The American Journal of Pathology. 1998 Nov;153(5):1425–33.

14. Manner J, Radlwimmer B, Hohenberger P, Mössinger K, Küffer S, Sauer C, et al. MYC High Level Gene Amplification Is a Distinctive Feature of Angiosarcomas after Irradiation or Chronic Lymphedema. The American Journal of Pathology. 2010 Jan;176(1):34–9.

15. Weihrauch M, Bader M, Lehnert G, Koch B, Wittekind C, Wrbitzky R, et al. Mutation analysis of K-ras-2 in liver angiosarcoma and adjacent nonneoplastic liver tissue from patients occupationally exposed to vinyl chloride. Environmental and Molecular Mutagenesis. Wiley Subscription Services, Inc., A Wiley Company; 2002 Jan 1;40(1):36–40.

16. Hayashi T, Koike K, Kumasaka T, Saito T, Mitani K, Terao Y, et al. Uterine angiosarcoma associated with lymphangioleiomyomatosis in a patient with tuberous sclerosis complex: an autopsy case report with immunohistochemical and genetic analysis. Human Pathology. 2012 Oct;43(10):1777–84.

17. Guo T, Zhang L, Chang N-E, Singer S, Maki RG, Antonescu CR. Consistent MYC and FLT4 gene amplification in radiation-induced angiosarcoma but not in other radiation-associated atypical vascular lesions. Genes Chromosomes Cancer. Wiley Subscription Services, Inc., A Wiley Company; 2011 Jan;50(1):25–33.

18. Murali R, Chandramohan R, Möller I, Scholz SL, Berger M, Huberman K, et al. Targeted massively parallel sequencing of angiosarcomas reveals frequent activation of the mitogen activated protein kinase pathway. Oncotarget. Impact Journals, LLC; 2015 Nov 3;6(34):36041–52.

19. Behjati S, Tarpey PS, Sheldon H, Martincorena I, Van Loo P, Gundem G, et al. Recurrent PTPRB and PLCG1 mutations in angiosarcoma. Nat Genet. Nature Research; 2014 Apr 1;46(4):376–9.

20. Landuzzi L, Ianzano ML, Nicoletti G, Palladini A, Grosso V, Ranieri D, et al. Genetic prevention of lymphoma in p53 knockout mice allows the early development of p53-related sarcomas. Oncotarget. Impact Journals; 2014 Dec 15;5(23):11924–38.

21. Ghahremani MF, Radaelli E, Haigh K, Bartunkova S, Haenebalcke L, Marine J-C, et al. Loss of autocrine endothelial-derived VEGF significantly reduces hemangiosarcoma development in conditional p53-deficient mice. Cell Cycle. Taylor & Francis; 2014 Mar 11;13(9):1501–7.

22. Papa A, Cordon-Cardo C, Bernardi R, Pandolfi PP. Compound In Vivo Inactivation of Pml and p53 Uncovers a Functional Interaction in Angiosarcoma Suppression. Genes Cancer. SAGE Publications; 2012 Sep;3(9-10):599–603.

23. Yang J, Kantrow S, Sai J, Hawkins OE, Boothby M, Ayers GD, et al. Ikk4a/Arf Inactivation with Activation of the NF-κB/IL-6 Pathway Is Sufficient to Drive the Development and Growth of Angiosarcoma. Cancer Res. American Association for Cancer Research; 2012 Sep 15;72(18):4682–95.

24. Italiano A, Chen C-L, Thomas R, Breen M, Bonnet F, Sevenet N, et al. Alterations of the p53 and PIK3CA/AKT/mTOR pathways in angiosarcomas: a pattern distinct from other sarcomas with complex genomics. Cancer. Wiley Subscription Services, Inc., A Wiley Company; 2012 Dec 1;118(23):5878–87.

25. Tate G, Suzuki T, Mitsuya T. Mutation of the PTEN gene in a human hepatic angiosarcoma. Cancer Genetics and Cytogenetics. 2007 Oct 15;178(2):160–2.

26. Leech JD, Lammers SHT, Goldman S, Auricchio N, Bronson RT, Kwiatkowski DJ, et al. A Vascular Model of Tsc1 Deficiency Accelerates Renal Tumor Formation with Accompanying Hemangiosarcomas. Mol Cancer Res. American Association for Cancer Research; 2015 Mar 1;13(3):548–55.

27. Dill MT, Rothweiler S, Djonov V, Hlushchuk R, Tornillo L, Terracciano L, et al. Disruption of Notch1 Induces Vascular Remodeling, Intussusceptive Angiogenesis, and Angiosarcomas in Livers of Mice. Gastroenterology. 2012 Apr;142(4):967–977.e2.

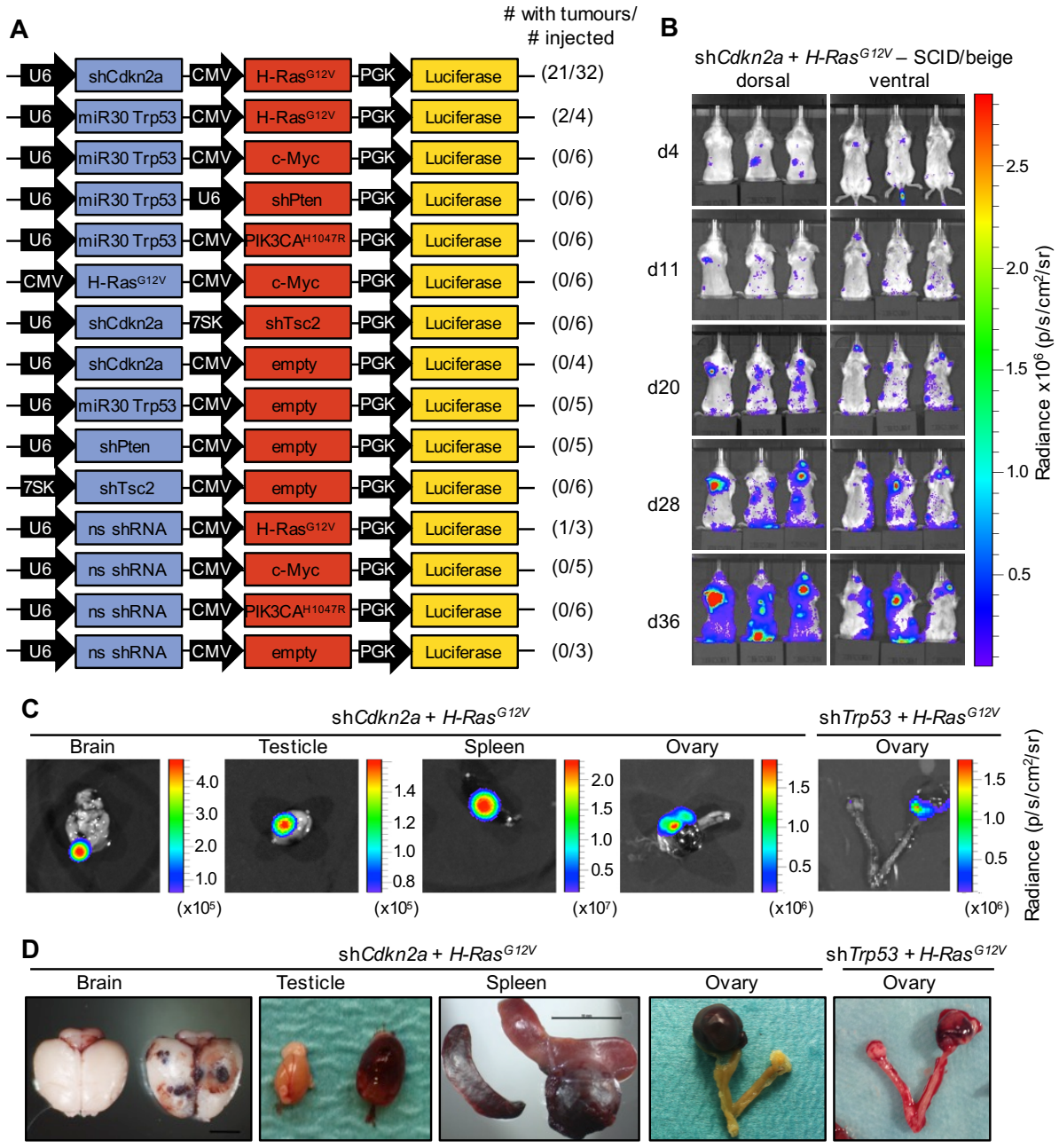


Figure 1. *H-Ras*^{G12V} expression plus knockdown of *Cdkn2a* or *Trp53* causes angiosarcomas in SCID/beige mice. (**A**) Schematic of MuLE vectors simultaneously expressing combinations of shRNAs against *Cdkn2a*, *Trp53*, *Pten* and *Tsc2* with or without expression of *H-Ras, c-Myc* and *PIK3CA* and a Luciferase reporter. (**B**) Bioluminescence imaging 4, 11, 20, 28 and 36 days after the injection of MuLE lentiviruses expressing a shRNA against *Cdkn2a* together with *H-Ras*^{G12V} into the tail vein of 4-6-week-old SCID/beige. Numbers of mice that developed tumours and total number of injected mice are shown for each construct. (**C**) Bioluminescence imaging and (**D**) photographs showing examples of tumour-bearing organs from sh*Cdkn2a* plus *H-Ras*^{G12V} and sh*Trp53* plus *H-Ras*^{G12V} injected animals. Scale bar: 10mm.

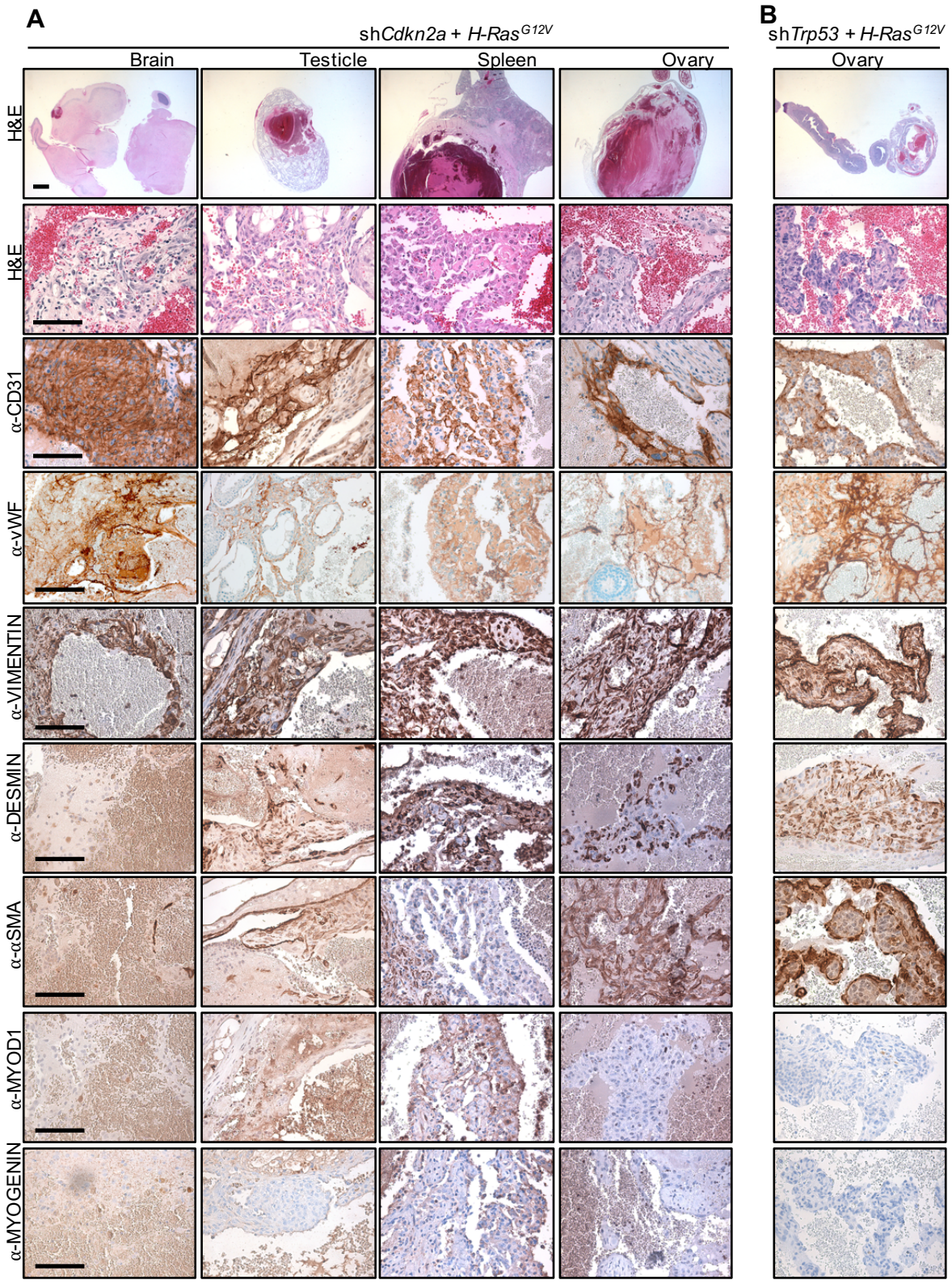


Figure 2. Histological and molecular characterisation of angiosarcomas.
H&E and immunohistochemical stainings using the indicated antibodies of tumours derived from the systemic injection of (**A**) sh*Cdkn2a* plus *H-Ras*^{G12V} and (**B**) sh*Trp53* plus *H-Ras*^{G12V} viruses. Low magnification scale bar: 1000 µm and high magnification scale bar: 100 µm.

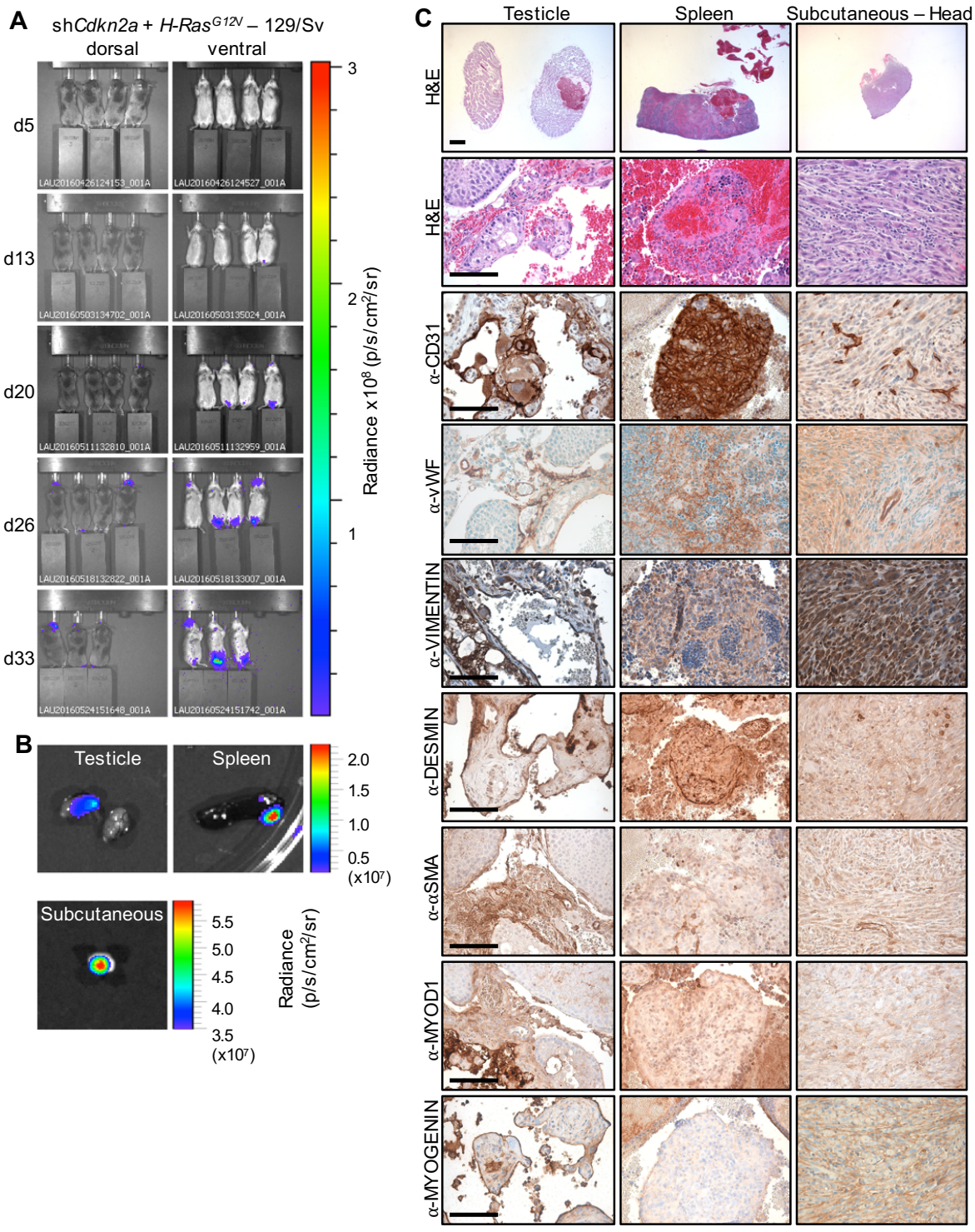


Figure 3. *H-Ras*^{G12V} expression plus knockdown of *Cdkn2a* causes angiosarcomas and undifferentiated pleomorphic sarcomas in 129Sv mice. (**A**) Bioluminescence imaging 5, 13, 20, 26 and 33 days after the injection of MuLE lentiviruses expressing a shRNA against *Cdkn2a* together with *H-Ras*^{G12V} into the tail vein of 4-6-week-old 129/Sv mice. (**B**) Bioluminescence imaging showing examples of tumour-bearing organs. (**C**) H&E and immunohistochemical stainings using the indicated antibodies. Low magnification scale bar: 1000 µm and high magnification scale bar: 100 µm.

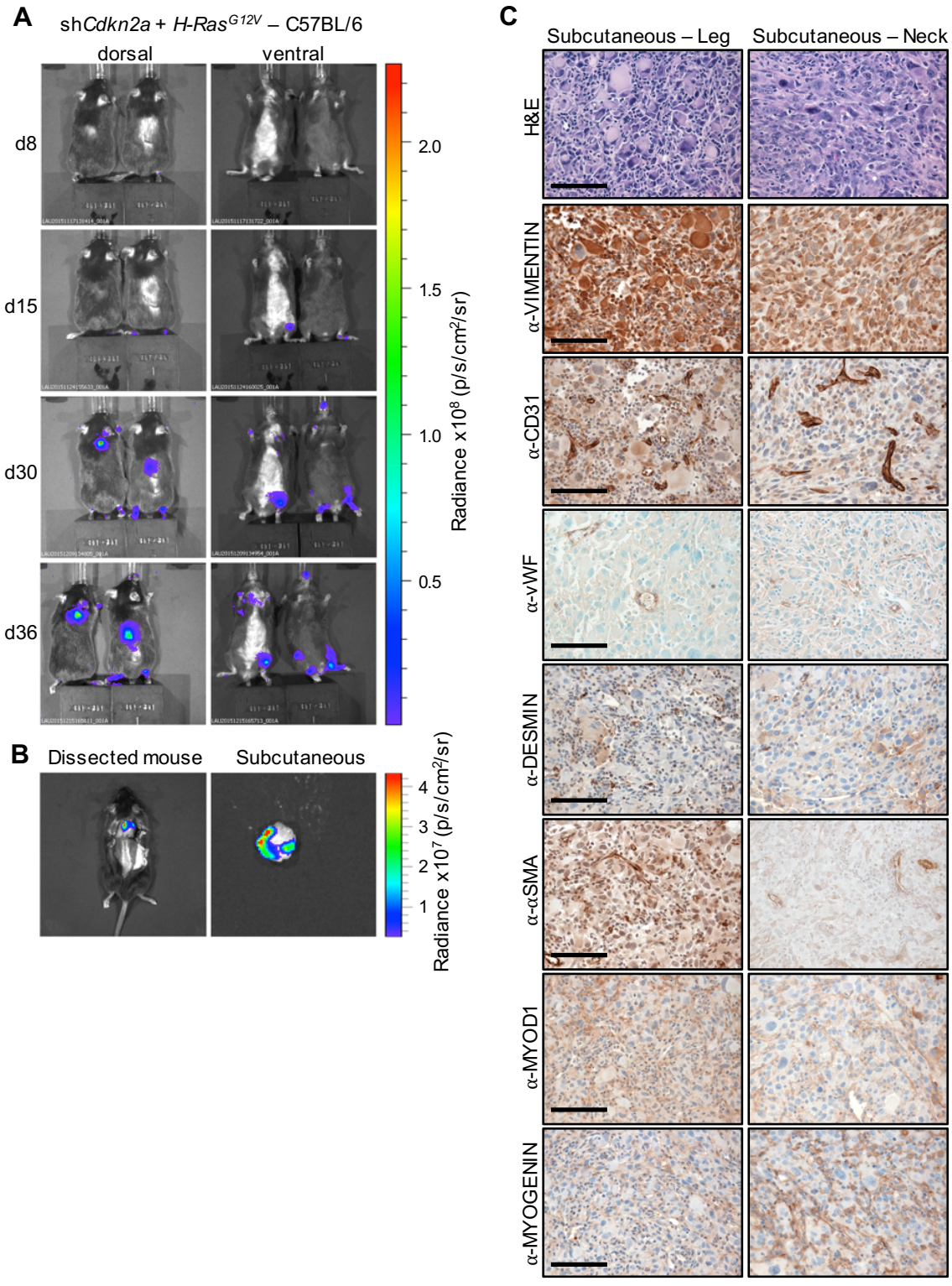
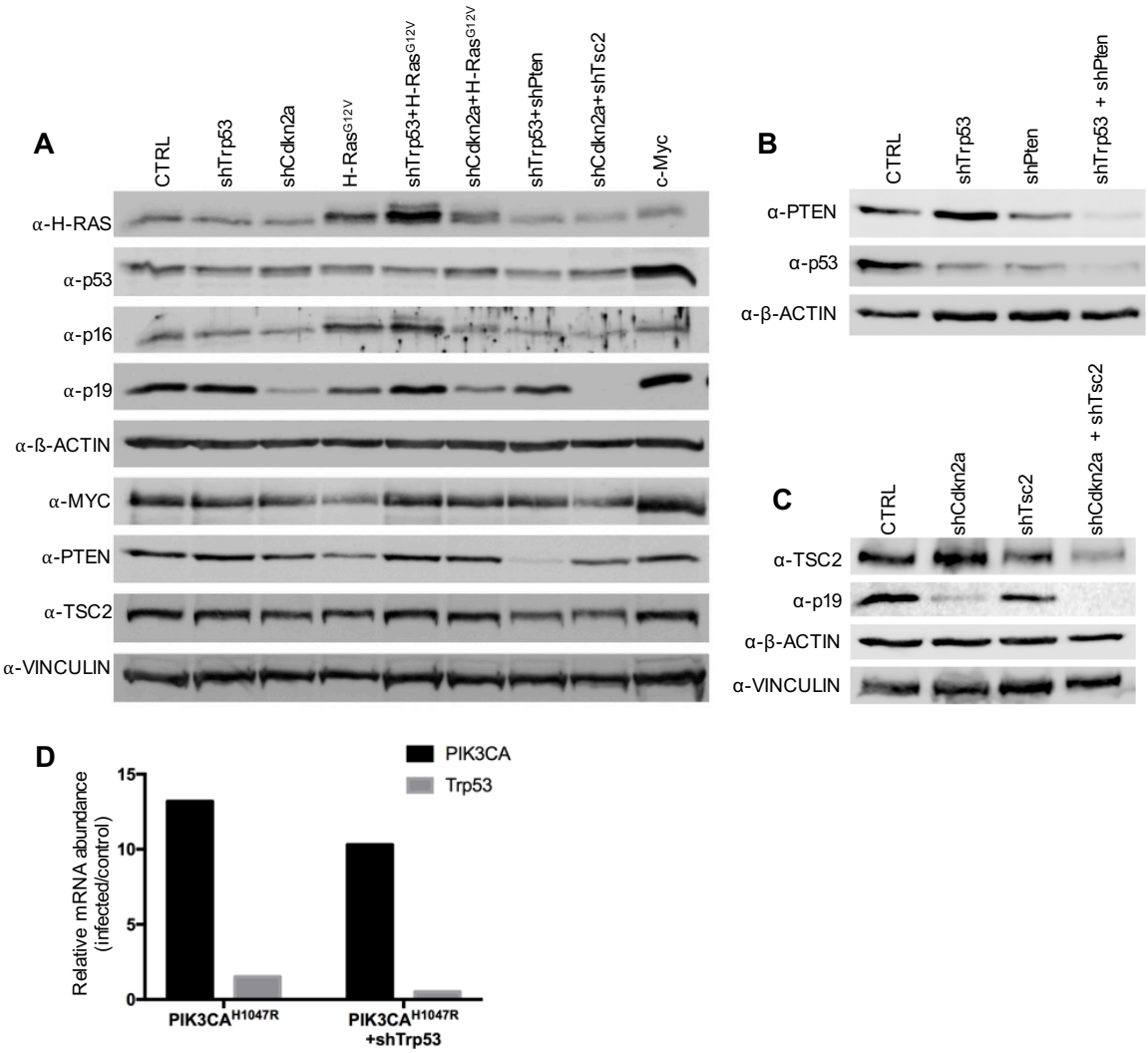
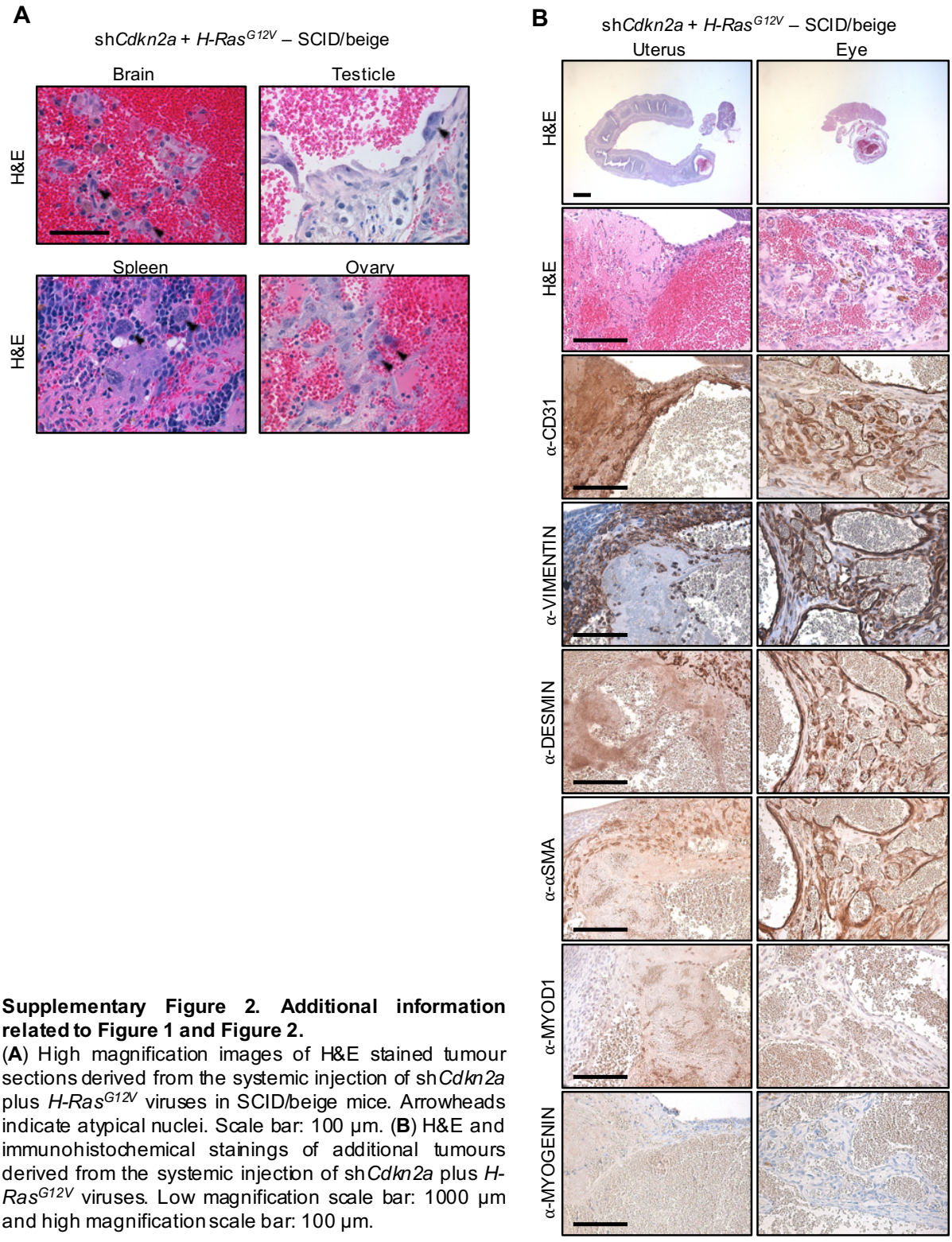


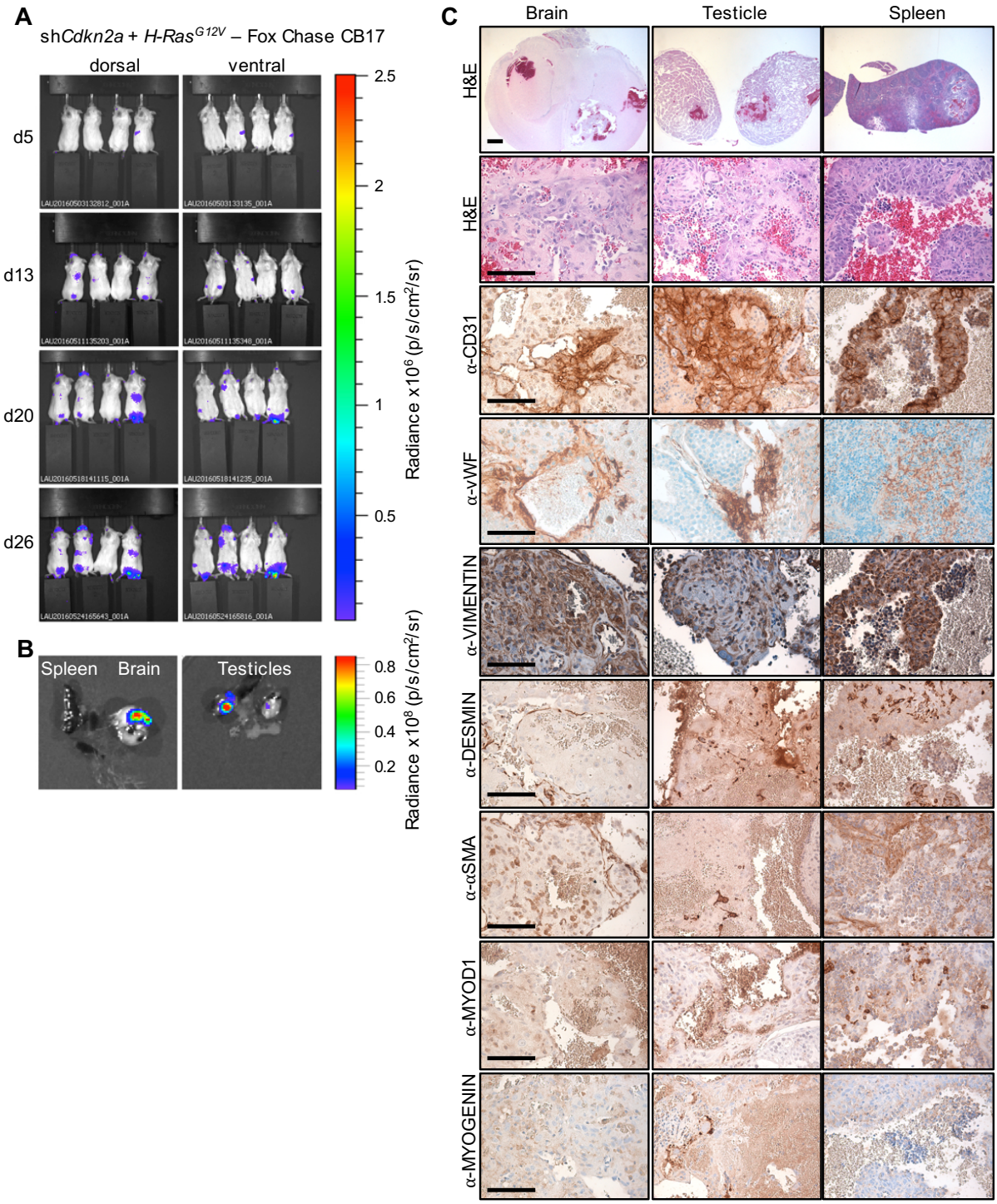
Figure 4. *H-Ras*^{G12V} expression plus knockdown of *Cdkn2a* causes undifferentiated pleomorphic sarcomas in C57Bl/6 mice. (**A**) Bioluminescence imaging 8, 15, 30 and 36 days after the injection of MuLE lentiviruses expressing a shRNA against *Cdkn2a* together with *H-Ras*^{G12V} into the tail vein of 4-6-week-old C57Bl/6mice. (**B**) Bioluminescence imaging showing examples of a subcutaneous tumour prior to (left) and after (right) resection. (**C**) H&E and immunohistochemical stainings using the indicated antibodies. Low magnification scale bar: 100 µm.



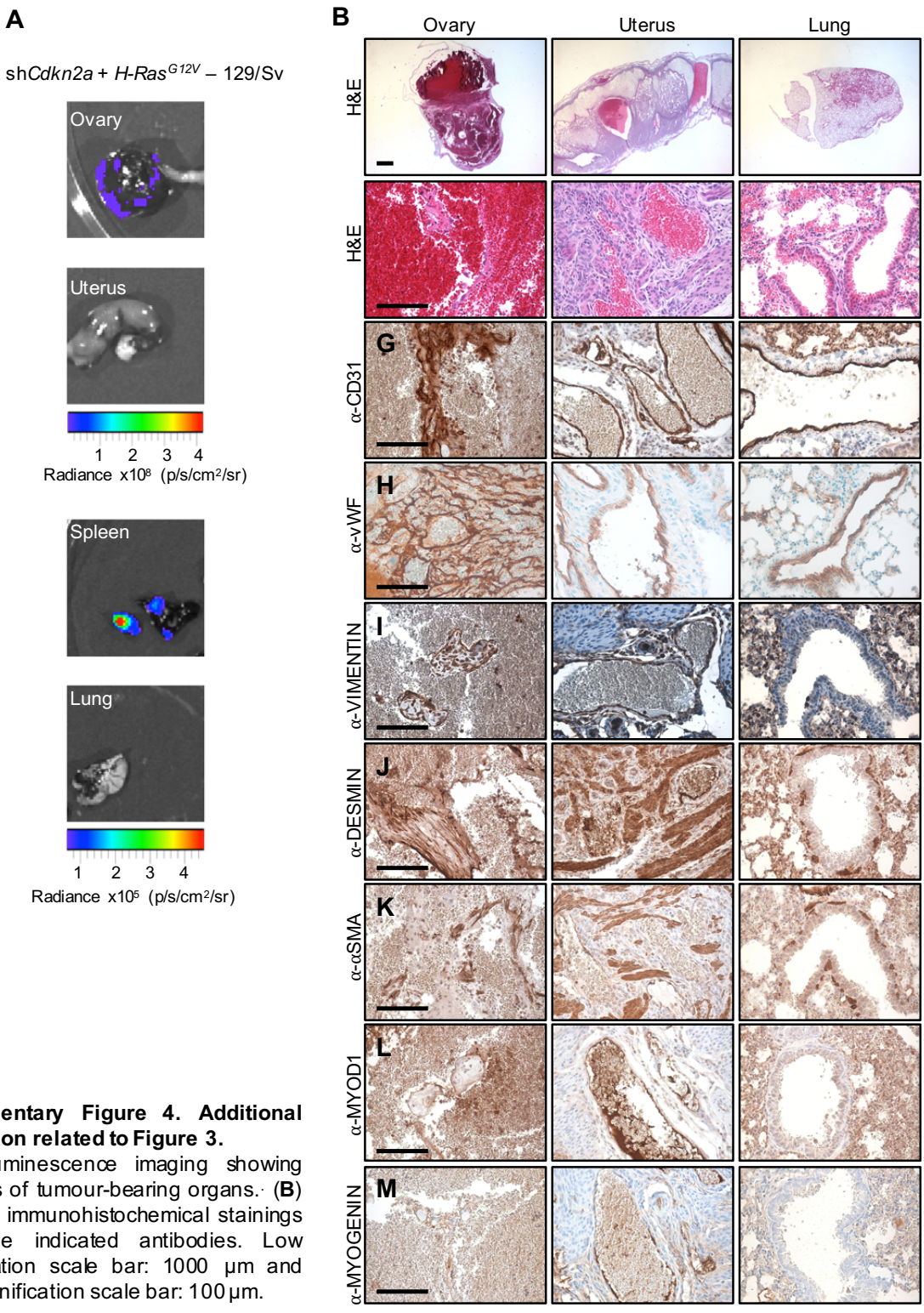
Supplementary Figure 1. Validation of MuLE vectors by western blot and real time PCR analysis. (**A**-**C**) Western blots of primary endothelial cells from the spleen (pMSECs) transduced with the indicated lentiviruses. (**D**) Relative gene expression of PIK3CA and Trp53 in pMSECs transduced with the indicated lentiviruses.



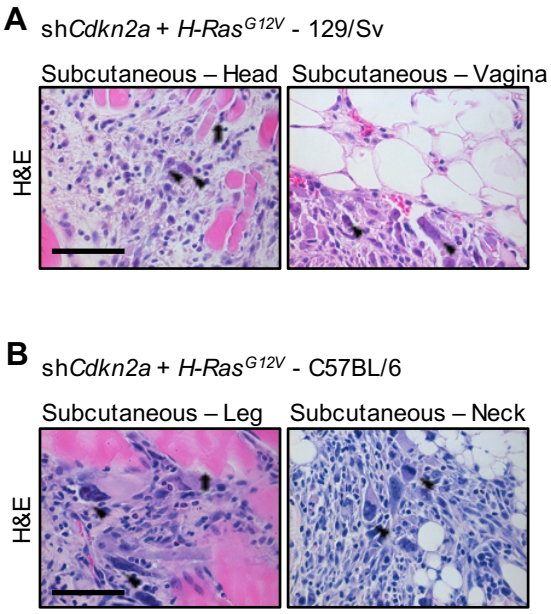
Supplementary Figure 2. Additional information related to Figure 1 and Figure 2.
(**A**) High magnification images of H&E stained tumour sections derived from the systemic injection of sh*Cdkn2a* plus *H-Ras*^{G12V} viruses in SCID/beige mice. Arrowheads indicate atypical nuclei. Scale bar: 100 µm. (**B**) H&E and immunohistochemical stainings of additional tumours derived from the systemic injection of sh*Cdkn2a* plus *H-Ras*^{G12V} viruses. Low magnification scale bar: 1000 µm and high magnification scale bar: 100 µm.



Supplementary Figure 3. *H-Ras*^{G12V} expression plus knockdown of *Cdkn2a* causes angiosarcomas in Fox Chase CB17 mice. (**A**) Bioluminescence imaging 5, 13, 20 and 26 days after the injection of MuLE lentiviruses expressing a shRNA against *Cdkn2a* together with *H-Ras*^{G12V} into the tail vein of 4-6-week-old Fox Chase CB17 mice. (**B**) Bioluminescence imaging showing examples of tumour-bearing organs. (**C**) H&E and immunohistochemical stainings using the indicated antibodies. Low magnification scale bar: 1000 µm and high magnification scale bar: 100 µm.



Supplementary Figure 4. Additional information related to Figure 3.
(**A**) Bioluminescence imaging showing examples of tumour-bearing organs. (**B**) H&E and immunohistochemical stainings using the indicated antibodies. Low magnification scale bar: 1000 µm and high magnification scale bar: 100 µm.



Supplementary Figure 5. High grade, locally invasive undifferentiated pleomorphic sarcomas form in 129/Sv and C57BL/6 mice.
High magnification images of H&E stained tumour sections derived from the intraveneous injection of sh*Cdkn2a* plus *H-Ras*^{G12V} viruses in (**A**) 129/Sv mice and (**B**) C57BL/6 mice. Arrowheads indicate atypical nuclei and arrows indicate entrapped muscle fibers. Images decipt tumour invasion into the surrounding muscle or fat tissue. Scale bar: 100 µm.

2.3 Results - Molecularly targeted therapies for angiosarcoma treatment

2.3.1 Generation and characterisation of genetically-defined angiosarcoma cell lines

To complement the described *in vivo* studies with *in vitro* tumour modelling studies we aimed to generate panels of genetically-defined transformed angiosarcoma cell lines. Since we discovered that the combination of *H-Ras*^{G12V} and sh*Cdkn2a* or sh*Trp53* is sufficient to initiate angiosarcoma development *in vivo*, we decided to generate genetically-defined angiosarcoma cell lines which reflect these changes. A disease-relevant cell type, namely primary murine endothelial cells from the spleen (pMSECs), were transduced with ecotropic lentiviruses expressing combinations of shRNA against *Cdkn2a* or shRNA-miR30 against *Trp53* with or without expression of *H-Ras*^{G12V} and puromycin resistance (Fig. 2.3.1A). Western blot analysis of cultured cells infected with these vectors verified that they effectively induced the desired changes in gene expression (Chapter 2.2, Supplementary Fig. 1).

pMSECs transduced with sh*Cdkn2a* and sh*Trp53* alone, as well as oncogenic *H-Ras* plus sh*Cdkn2a* or sh*Trp53* showed an increased proliferation rate compared to wild type pMSECs. *H-Ras*^{G12V} transduced pMSECs showed similar growth kinetics to wild type cells (Fig. 2.3.1B). This observation is consistent with the fact that the expression of oncogenic *Ras* in some types of primary cells causes permanent G1 arrest and that the cooperation of another oncogene or the inactivation of tumour suppresors, such as p53 or p16 is required for oncogenic transformation (245).

To further investigate cancer-relevant aspects of cellular behaviour, we tested the capacity of these cells to grow in an anchorage independent manner, a common characteristic of transformed primary cells or of tumour cell lines. Cells were seeded at low density in cell culture dishes with a hydrophobic surface, which minimizes cell attachment. All the different cell lines were unable to form spheroids (data not shown), showing that they do not have anchorage-independent proliferation capacity.

We next studied the tumour-forming capacity of sh*Cdkn2a* plus *H-Ras*^{G12V} transduced cells. 1x10^6 pMSECs (wild type and sh*Cdkn2a* plus *H-Ras*^{G12V}) were subcutaneously

injected in immunodeficient SCID/beige. Within 31 days the mice developed blood-filled lesions (Fig. 2.3.1C). To our surprise, both wild type and sh*Cdkn2a* plus oncogenic *H-Ras* expressing cells were able to form these lesions. The lesions were comprised of malignant CD31-positive endothelial cells with atypical nuclei growing either as single layers of cells or in papillary projections. The injected cells apparently have the capacity to co-opt or integrate into local blood vessels, resulting in large, blood-filled vascular structures. Based on the fact that wild type pMSECs showed tumour-initiating properties without the need of oncogene activation or tumour suppressor inactivation, we decided to not continue with this alternative research strategy.

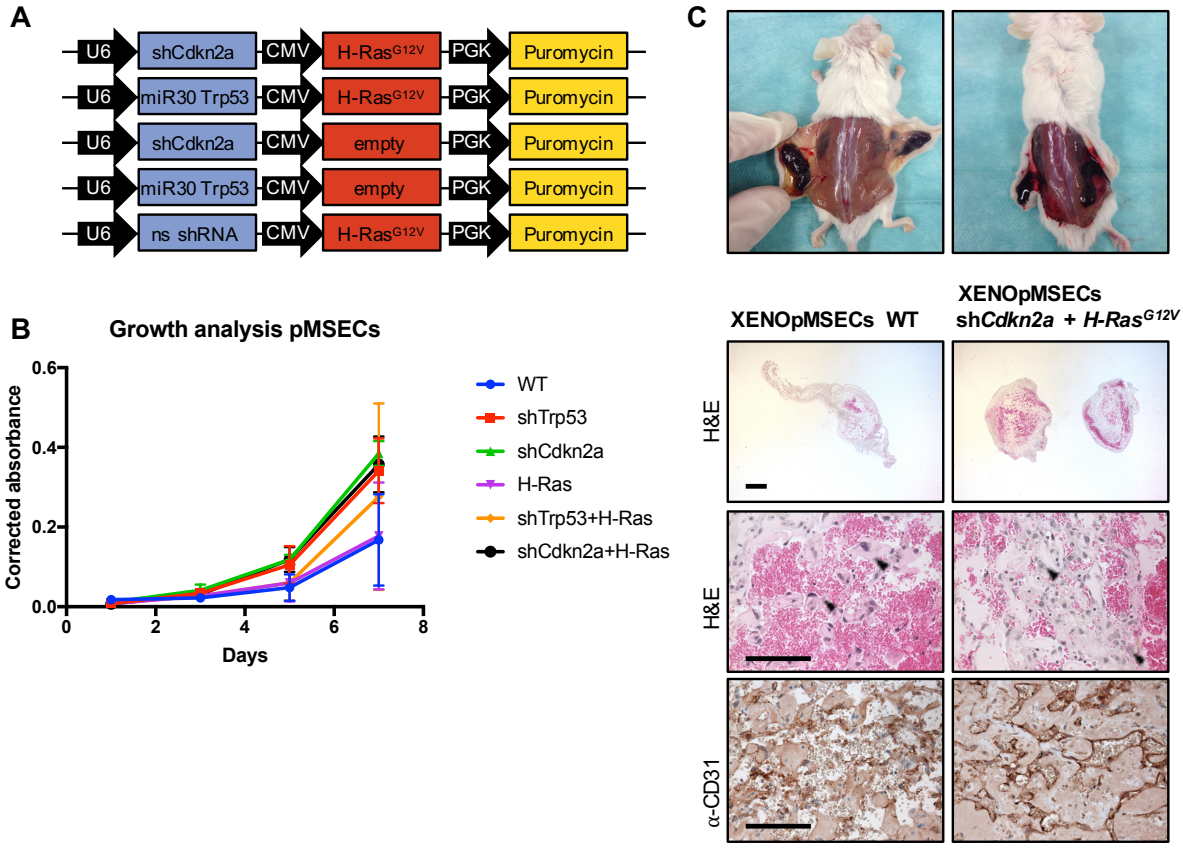
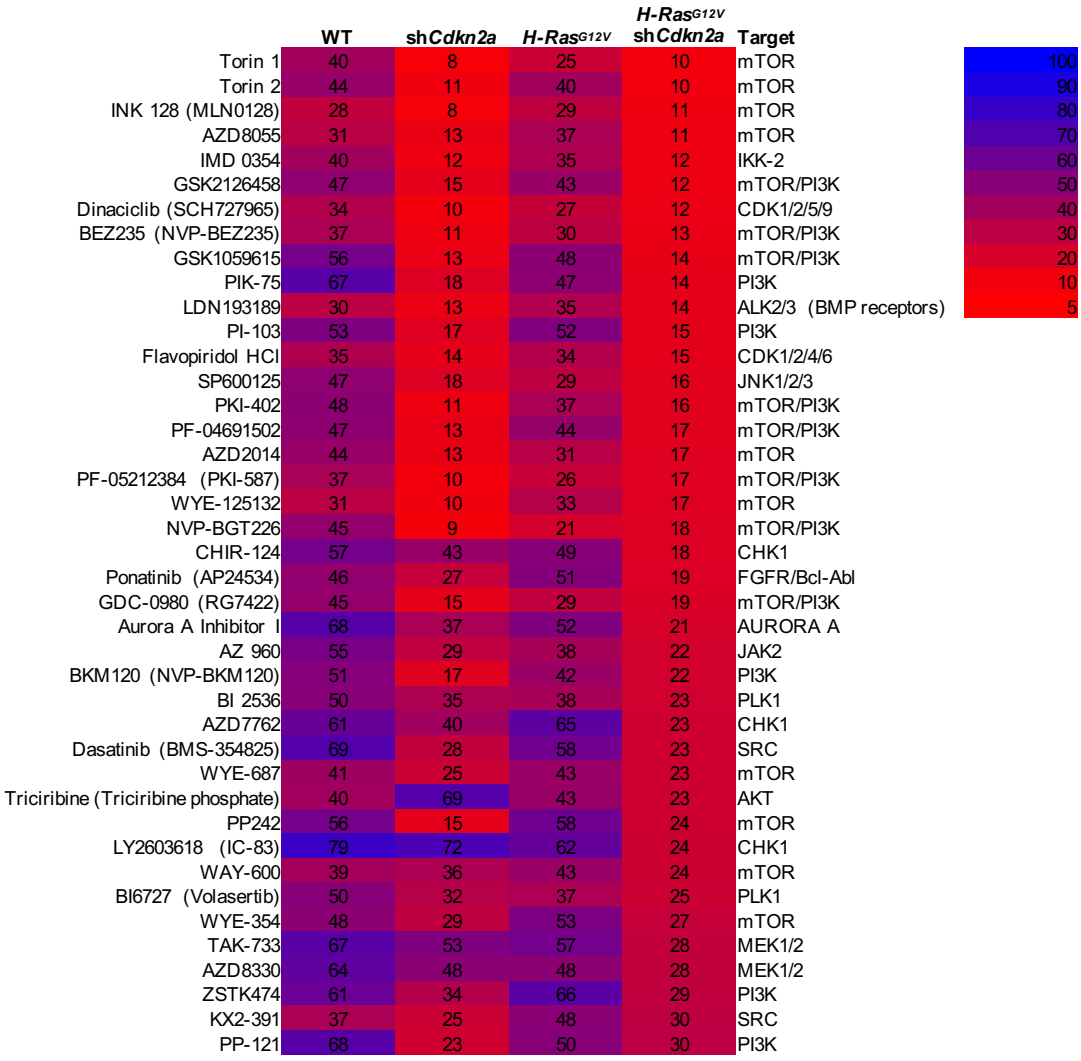


Figure 2.3.1. Generation and characterisation of genetically-defined angiosarcoma cell lines. (**A**) Schematic of MuLE vectors simultaneously expressing combinations of shRNAs against *Cdkn2a* and *Trp53* with or without expression of *H-Ras*^{G12V} and puromycin resistance. (**B**) Growth analysis after 1, 3, 5 and 7 days of pMSECs transduced with the afore-mentioned lentiviral vectors. (**C**) Growth of cells as tumour xenografts and images of tumors derived from WT and sh*Cdkn2a* plus *H-Ras*^{G12V} transduced pMSECs. Low magnification scale bar: 1000 µm and high magnification scale bar: 100 µm.

2.3.2 Cytotoxicity screening of a panel of a kinase inhibitor library

Several clinical trials are testing therapeutic agents directed against signalling molecules which are frequently altered in human angiosarcomas. A significant disadvantage of these trials however, is that almost without exception, they are not being coupled to the analysis of the underlying molecular alterations in each individual patient. Therefore, we aimed to test the cytotoxic effects of 273 kinase inhibitors, which target a broad range of kinases, on the previously established *Cdkn2a* silenced and activated *H-Ras*-expressing endothelial cell line with the idea that we might be able to uncover therapeutic sensitivities that are dependent on the defined genetics of each cell line. Wild type, oncogenic *H-Ras*, sh*Cdkn2a* and sh*Cdkn2a* plus oncogenic *H-Ras* transduced pMSECs were seeded in 96 well plates and chronically treated with a 1µM concentration of the library inhibitors for 96 hours. The SRB assay was used for cell density determination. The percentage of cell growth inhibition was calculated by comparing the values of inhibitor-treated cells to untreated cells.

41 drugs reduced growth to 30% or less compared to control cell growth of sh*Cdkn2a* plus *H-Ras*^{G12V} transduced pMSECs (Fig. 2.3.2). The most commonly targeted pathway was the PI3K/mTOR signalling network. Inhibitors targeting this pathway significantly reduced protein content in both sh*Cdkn2a* driven cell lines while they had minimal effects on the protein content of wild type and *H-Ras*^{G12V} expressing cell lines. Additionally, the screen revealed that cells expressing oncogenic *H-Ras* together with the knockdown of *Cdkn2a* are highly sensitive to CHK1 pathway inhibitors. To further determine whether the introduced genetic changes of our mutant *H-Ras* and sh*Cdkn2a* expressing cell line influence the response to the drugs, we performed drug-response analysis with selected inhibitors. Inhibitors which reduced growth to at least 23% compared to untreated control and which specifically targeted our oncogenic *H-Ras* plus sh*Cdkn2a* expressing endothelial cells were chosen for further analyses.



	WT	sh*Cdkn2a*	*H-Ras*^{G12V}	*H-Ras*^{G12V} sh*Cdkn2a*	Target
Torin 1	40	8	25	10	mTOR
Torin 2	44	11	40	10	mTOR
INK 128 (MLN0128)	28	8	29	11	mTOR
AZD8055	31	13	37	11	mTOR
IMD 0354	40	12	35	12	IKK-2
GSK2126458	47	15	43	12	mTOR/PI3K
Dinaciclib (SCH727965)	34	10	27	12	CDK1/2/5/9
BEZ235 (NVP-BEZ235)	37	11	30	13	mTOR/PI3K
GSK1059615	56	13	48	14	mTOR/PI3K
PIK-75	67	18	47	14	PI3K
LDN193189	30	13	35	14	ALK2/3 (BMP receptors)
PI-103	53	17	52	15	PI3K
Flavopiridol HCl	35	14	34	15	CDK1/2/4/6
SP600125	47	18	29	16	JNK1/2/3
PKI-402	48	11	37	16	mTOR/PI3K
PF-04691502	47	13	44	17	mTOR/PI3K
AZD2014	44	13	31	17	mTOR
PF-05212384 (PKI-587)	37	10	26	17	mTOR/PI3K
WYE-125132	31	10	33	17	mTOR
NVP-BGT226	45	9	21	18	mTOR/PI3K
CHIR-124	57	43	49	18	CHK1
Ponatinib (AP24534)	46	27	51	19	FGFR/Bcl-Abl
GDC-0980 (RG7422)	45	15	29	19	mTOR/PI3K
Aurora A Inhibitor I	68	37	52	21	AURORA A
AZ 960	55	29	38	22	JAK2
BKM120 (NVP-BKM120)	51	17	42	22	PI3K
BI 2536	50	35	38	23	PLK1
AZD7762	61	40	65	23	CHK1
Dasatinib (BMS-354825)	69	28	58	23	SRC
WYE-687	41	25	43	23	mTOR
Triciribine (Triciribine phosphate)	40	69	43	23	AKT
PP242	56	15	58	24	mTOR
LY2603618 (IC-83)	79	72	62	24	CHK1
WAY-600	39	36	43	24	mTOR
BI6727 (Volasertib)	50	32	37	25	PLK1
WYE-354	48	29	53	27	mTOR
TAK-733	67	53	57	28	MEK1/2
AZD8330	64	48	48	28	MEK1/2
ZSTK474	61	34	66	29	PI3K
KX2-391	37	25	48	30	SRC
PP-121	68	23	50	30	PI3K

Figure 2.3.2. Cytotoxicity screening of a panel of 273 different kinase inhibitors using genetically-defined endothelial cell lines. RTK inhibitor screening against pMSECs expressing combinations of shRNA against *Cdkn2a* with or without expression of *H-Ras*^{G12V} using the SRB assay in a 96 well format. Heat map of 41 drugs which reduced growth to 30% or less compared to control cell growth of sh*Cdkn2a* plus *H-Ras*^{G12V} transduced pMSECs: 10 mTOR inhibitors, 8 mTOR/PI3K inhibitors, 4 PI3K inhibitors, 3 CHK1 inhibitors, 2 CDK inhibitors, 2 SRC inhibitors, 2 PLK1 inhibitors, 2 MEK1/2 inhibitors, 1 IKK-2 inhibitor, 1 ALK2/2 inhibitor, 1 JNK inhibitor, 1 FGFR inhibitor, 1 Aurora A kinase inhibitor, 1 JAK2 inhibitor and 1 AKT inhibitor.

2.3.3 Drug-response analysis of selected PI3K and CHK1 inhibitors

Inhibitors targeting PI3K, Aurora A kinase and CHK1 were used for more detailed dose-response analyses. We selected the four CHK1 inhibitors LY2603618, AZD7762, CHIR-124 and PF477736, the three PI3K inhibitors NVP-BEZ235, PIK-75 and PI-103 and the Aurora A kinase inhibitor I. pMSECs expressing combinations of shRNA against *Cdkn2a* with or without expression of *H-Ras*^{G12V} were chronically treated with varying concentrations of the inhibitors for 96 hours and the SRB assay was used for

cell density determination. The percentage of cell growth inhibition was calculated by comparing the values of compound-treated cells to untreated cells. For IC_{50} determination, a dose-response curve was plotted between the inhibitor concentration and percent growth inhibition. IC_{50} values were derived using a curve-fitting method. These analyses revealed that the different cell lines were sensitive to all the tested PI3K inhibitors (Fig. 2.3.3A-C). The sensitivity ranged from an IC_{50} of 28 nM (NVP-BEZ235, Fig. 2.3.3A) to 100 nM (PIK-75, Fig. 2.3.3B). Furthermore, we observed a broad range of sensitivity to the tested CHK1 inhibitors from sensitive (IC_{50} 0.1µM, AZD7762, Fig. 2.3.4B) to less sensitive (IC_{50} 4.5 µM, PF477736, Fig. 2.3.4D). However, drug-response analyses revealed that there was no clear tendency for the cells with *H-Ras* and *Cdkn2a* gene alterations to be more sensitive to the inhibitors than those with wild type genes. The tested compounds inhibited endothelial cell proliferation independently of the introduced genetic changes and are therefore not suitable for the treatment of *H-Ras*-driven sarcomagenesis.

Since single-agent treatment failed to show efficacy in our model we tested the inhibitory effect of the combination of two CHK1 inhibitors, LY2603618 and CHIR-124 (Fig. 5A and B), as well as LY2603618 plus the PI3K inhibitor PIK-75 (Fig. 2.3.5C and D). We treated the cells with varying concentrations of one inhibitor in the presence of a fixed concentration of the second inhibitor. The fixed concentration was based on the IC_{50} value of shCdkn2a plus H-Ras^{G12V} expressing cells from the dose-response analysis of the single agents. We did not observe an increased tendency for the cells with *H-Ras* and *Cdkn2a* gene alterations to be more sensitive to the inhibitors than those with wild type genes.

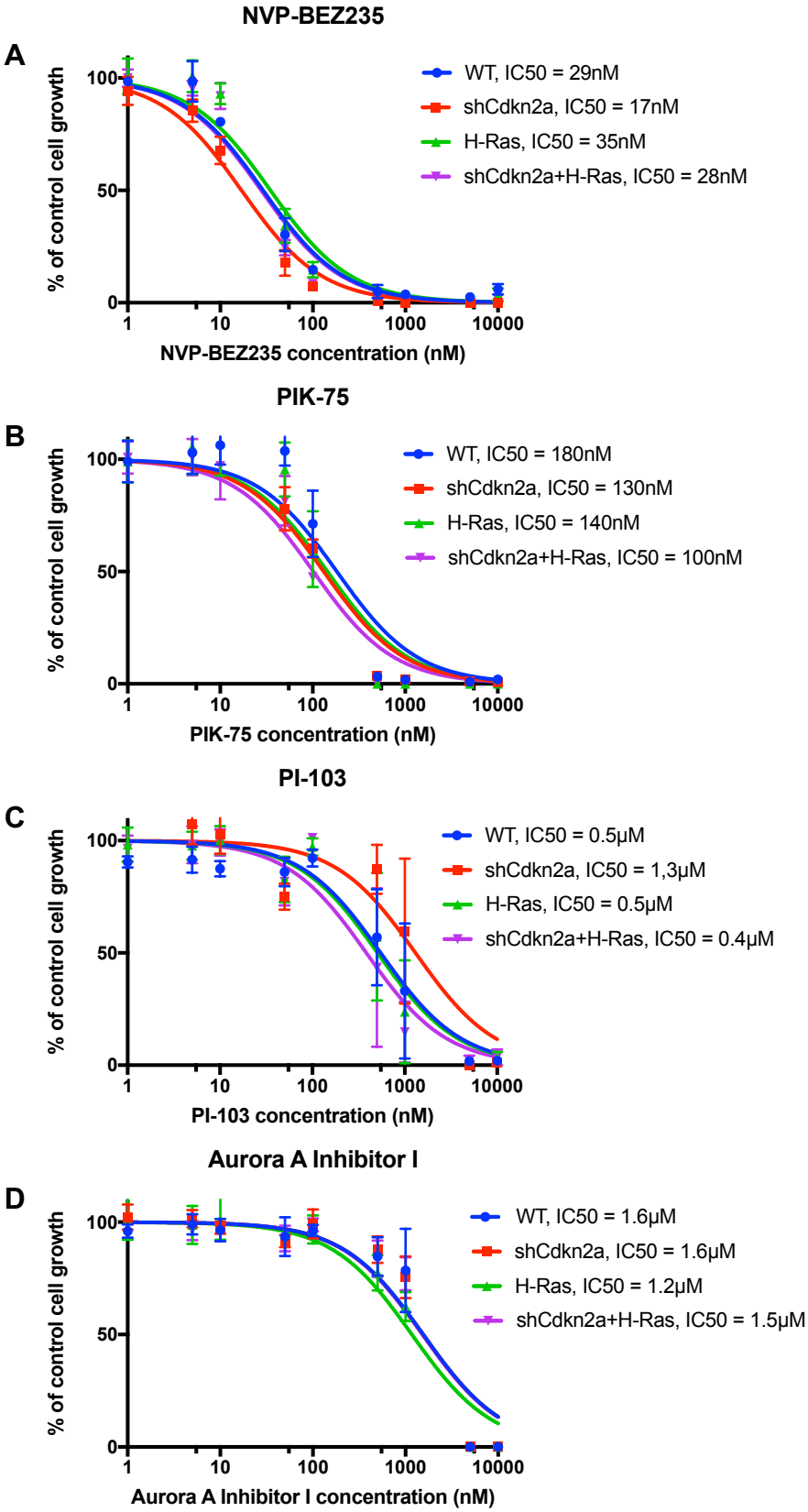


Figure 2.3.3. Dose-response analysis of three selected PI3K inhibitors, namely NVP-BEZ235, PIK-75 and PI-103 and the Aurora A kinase inhibitor I against endothelial cell lines expressing combinations of shRNA against *Cdkn2a* with or without expression of *H-Ras*^{G12V} using the SRB assay in a 96 well format.

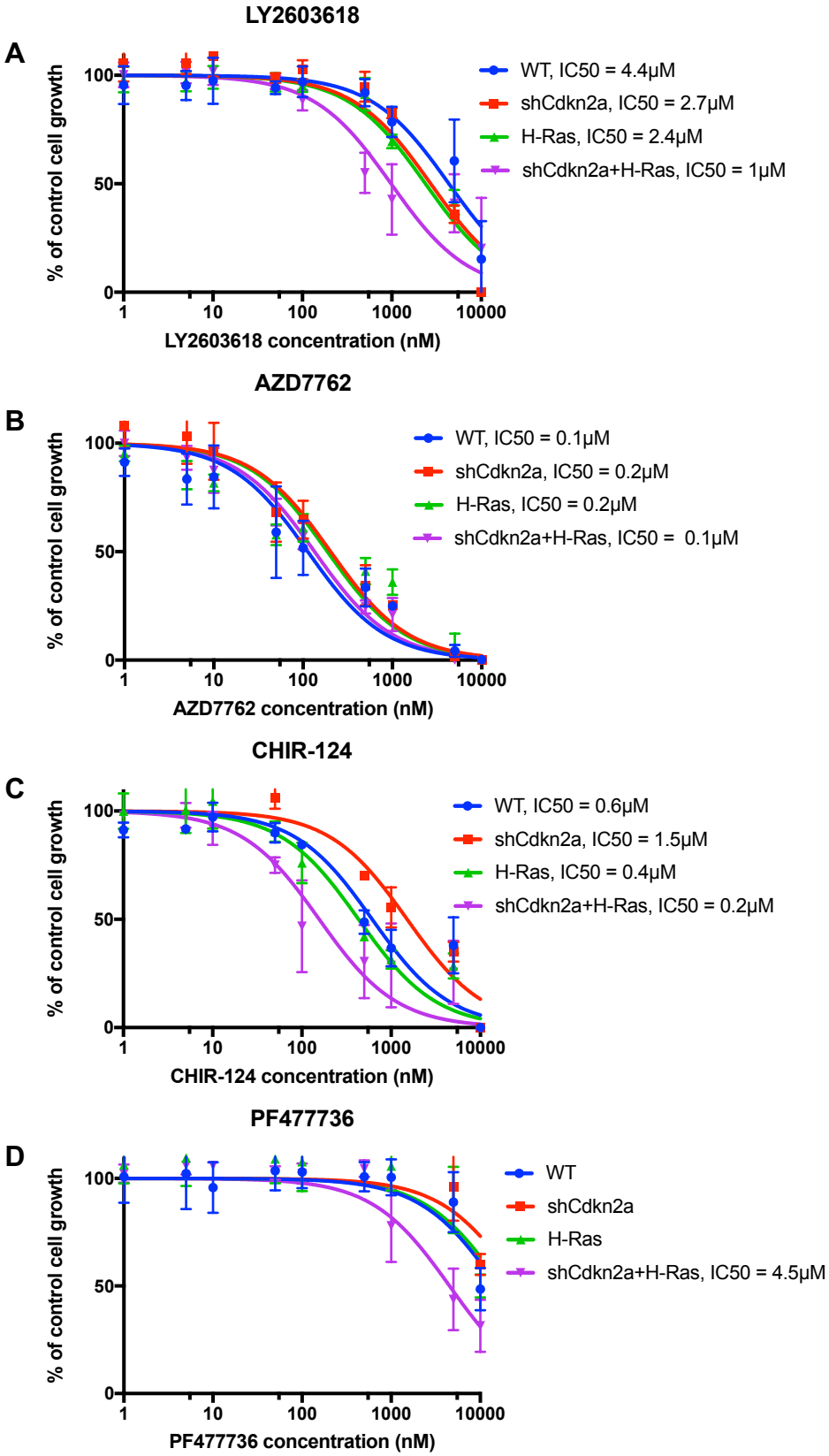


Figure 2.3.4. Dose-response analysis of four selected CHK1 inhibitors, namely LY2603618, AZD7762, CHIR-124 and PF477736 against endothelial cell lines expressing combinations of shRNA against *Cdkn2a* with or without expression of *H-Ras*^{G12V} using the SRB assay in a 96 well format.

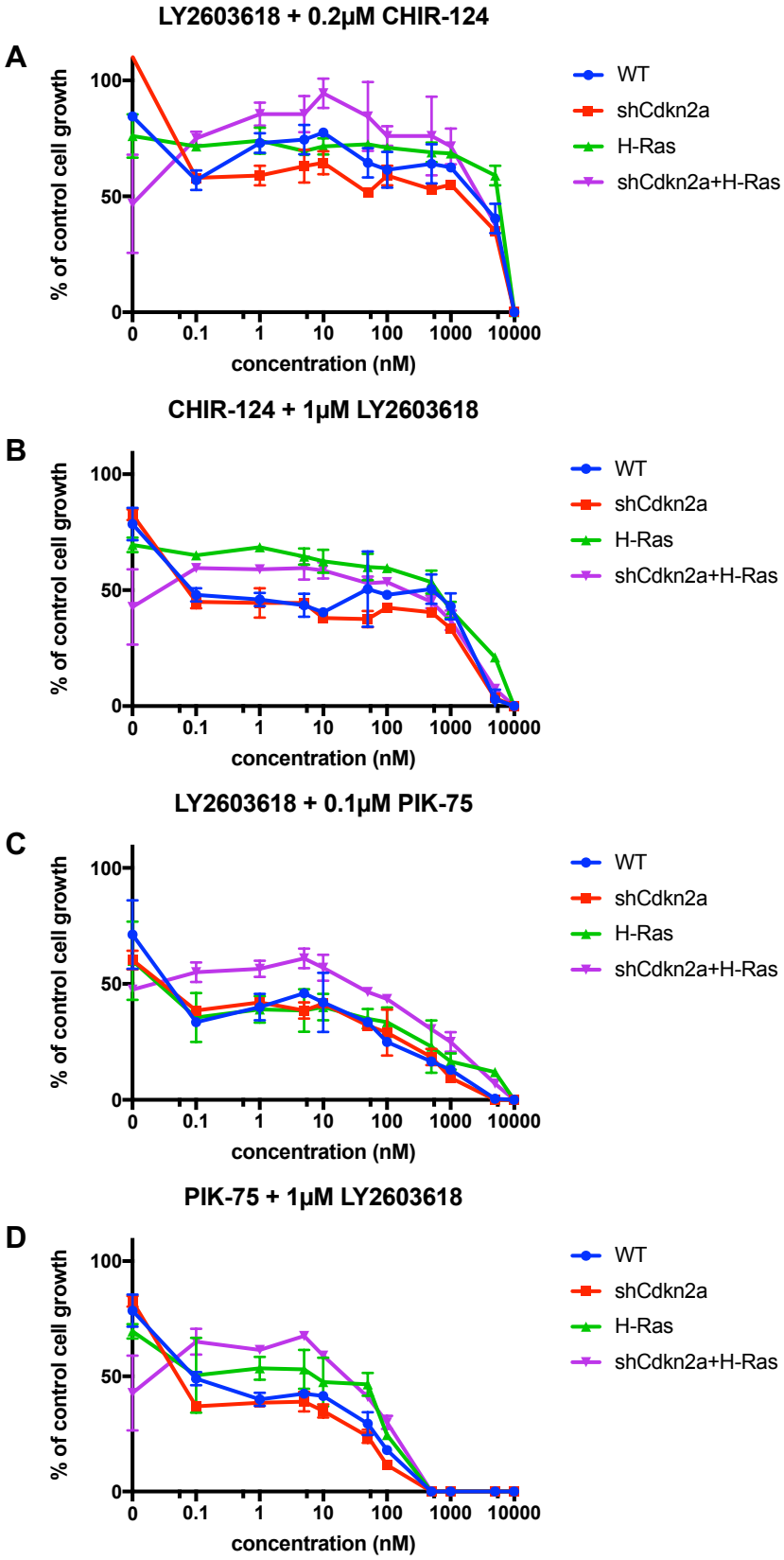


Figure 2.3.5. Dose-response analysis of drug combinations: Two CHK1 inhibitor LY2603618 and CHIR-124 as well as LY2603618 and the PI3K inhibitor PIK-75 against endothelial cell lines expressing combinations of shRNA against *Cdkn2a* with or without expression of *H-Ras*^{G12V} using the SRB assay in a 96 well format.

2.3.4 *In vivo* drug testing

Taking advantage of our monitorable, genetically-defined shRNA-*Cdkn2a* plus *H-Ras*^{G12V} driven angisarcoma mouse model which incorporates some of the known driver mutations and pathways involved in sarcomagenesis, we asked whether this model could be used for the identification and testing of appropriate therapies specific for these combinatorial mutations. We decided to test two inbitors targeting the MAPK and PI3K signalling pathways. AZD6244 (Selumetinib) is an orally bioavailable small molecule that inhibits MEK1/2, preventing the activation of MEK1/2-dependent effector proteins and transcription factors (246) and NVP-BEZ235 is a dual PI3K/mTOR inhibitor which spefically blocks the dysregulated activation of the PI3K/mTOR pathway and thereby induces G1 arrest (247).

We intraveneously injected SCID/beige mice with sh*Cdkn2a* plus oncogenic *H-Ras* expressing lentiviruses. Once the lesions reached a bioluminescence signal intensity of 10^{10} p/s (approximately 14-28 days), mice were treated daily with either vehicle, 50 mg/kg AZD6244 or 35 mg/kg NVP-BEZ235. These doses have been described to be therapeutically effective in a lung cancer mouse model driven by activation of *H-RAS* and *PIK3CA* (248). Mice were sacrificed when they reached a bioluminescence signal intesity of > 10^{12} p/s (Fig. 2.3.6A).

We found that chronic dosing with 50 mg/kg/d AZD6244 or 35 mg/kg/d NVP-BEZ235 did not inhibit tumour growth in our model. Median survival of vehicle-treated animals was 33 days (vehicle for NVP-BEZ235) and 39 days (vehicle for AZD6244) and compound treatment failed to enhance the survival (NVP-BEZ235 = 33 days and AZD6244 = 39 days) (Fig. 2.3.6B and C). Furthermore, four out of six NVP-BEZ235-treated mice had to be sacrificed due to a blocked up gastrointestinal tract. Tumour-bearing organs from the different drug-treated cohorts were harvested for histological and immunohistochemical assessment of PI3K-mTOR (p-S6 and p-4E-BP1) and RAS-MAPK (p-ERK) pathway activation. No differences between vehicle and inhibitor-treated animals were observed (Fig. 2.3.6D). We conclude that these therapeutic regimes are not likely to be suitable for the treatment of Ras-driven human angiosarcomas.

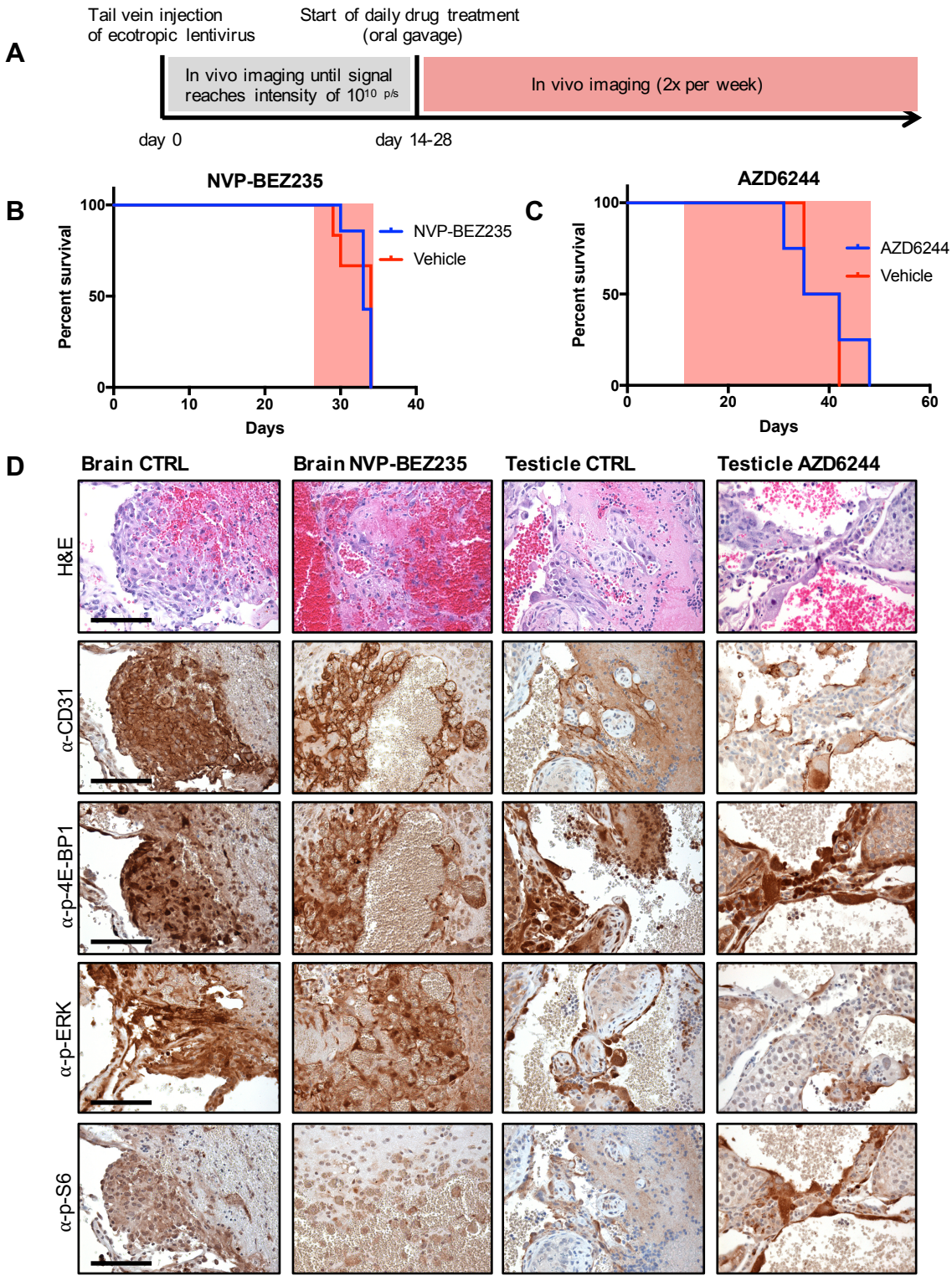


Figure 2.3.6. *In vivo* drug testing. (**A**) Schematic of treatment plan. (**B** and **C**) Overall survival of sh*Cdkn2a* plus *H-Ras*^{G12V} injected SCID/beige mice treated with NVP-BEZ235 (**B**) and AZD6244 (**C**). (**D**) H&E and immunohistochemical stainings using the indicated antibodies. Scale bar: 100 µm.

2.3.5 Methods - Molecularly targeted therapies for angiosarcoma treatment

2.3.5.1 Primary endothelial cell culture

C57BL/6 mouse primary spleen endothelial cells (pMSECs, C57-6057, Cell Biologics) were cultured according to the instructions of the supplier in complete mouse endothelial cell medium (M1168, Cell Biologics) in a humidified 5% (v/v) CO_2 incubator (Nuaire) at 37 °C.

2.3.5.2 Cell transduction

Endothelial cells were transduced with lentiviral vectors in the presence of polybrene (Hexadimethrine bromide, 107689, Sigma-Aldrich) (4 µg/mgl) at a density of 50%. 48h after lentiviral infection puromycin (ANT-PR-1, InvivoGen) (4 µg/mgl) was added to the cells. Treatment was continued until all the control cells were dead.

2.3.5.3 Proliferation assay

pMSECs were seeded at a density of 2000 cells **per well in 96 well plates and analysed after 1, 3, 5 and 7 days using the SRB assay** (249) **and the Infinite® 200 PRO microplate reader (Tecan). Every experiment** was performed in triplicates.

2.3.5.4 Drug screening

Cells were seeded at a density of 2000 cells per well in 96 well plates. 48h after the seeding cells were treated with 1 µM of the kinase inhibitor screening library (L1200, Selleckchem) for 96h. Medium and inhibitor were changed every day with the BIOMEK FX^P liquid handling workstation (Beckman Coulter). After completion of the drug exposure, plates were analysed using the sulforhodamine B (SRB) colorimetric assay and Infinite® 200 PRO microplate reader (Tecan). The relative protein content in each well was determined and normalized to an untreated control.

2.3.5.5 Drug-response analysis

Cells were seeded at a density of 2000 cells per well in 96 well plates. 48h after the seeding cells were treated with varying concentrations (0.1 nM, 1 nM, 5 nM, 10 nM, 50 nM, 0.1 µM, 0.5 µM, 1 µM, 5 µM and 10 µM) of the different kinase inhibitors for 96h. After completion of the drug exposure, plates were analysed using the SRB assay and Infinite® 200 PRO microplate reader (Tecan). The relative protein content in each well was determined and normalized to an untreated control.
Inhibitors used were Aurora A Inhibitor I (S1451, Selleckchem), AZD7762 (S1532, Selleckchem), CHIR-124 (S2683, Selleckchem), LY2603618 (S2626, Selleckchem), NVP-BEZ235 (S1009, Selleckchem), PF477736 (S2904, Selleckchem), PI-103 (S1038, Selleckchem) and PIK-75 (S1205, Selleckchem), all dissolved in dimethyl sulfoxide (DMSO) (D1435, Sigma Aldrich). Final DMSO concentration was kept at 0.1% in control and inhibitor-treated cells.

2.3.5.6 *In vivo* drug testing

10 ml/kg of concentrated ecotropic lentiviruses were injected through a 30G insulin syringe into the lateral tail vein of 4-6-week old mice. Noninvasive *in vivo* bioluminescence imaging was performed using the IVIS Spectrum (Perkin Elmer) together with the Living Image software (version 4.4). Once the lesions reached a bioluminescence signal intensity of 10^{10} p/s, 6 mice in each of the cohorts were treated via oral gavage with either vehicle, 50 mg/kg/d AZD6244 or 35mg/kg/d NVP-BEZ235. AZD6244 (S1008, Selleckchem) was dissolved in 0.5% Methylcellulose (M0512, Sigma Aldrich)/ 0.2% Tween-80 (P8074, Sigma Aldrich) in H_2O. The final working concentration was 30 mg/ml. NVP-BEZ235 (S1009, Selleckchem) was dissolved in NMP (32864, Sigma Aldrich)/Polyethylene glycol 300 (202398, Sigma Aldrich) (10/90, v/v). Solutions (5mg/ml) were prepared freshly every day. Mice weight was measured daily and tumour burden was measured twice a week by *in vivo* bioluminescence imaging. Endpoint criteria were a bioluminescence signal intesity of > 10^{12} p/s, decreasing body weight, poor body condition as well as unresponsive behaviour.

2.3.5.7 Immunohistochemistry

Tumour-bearing organs were resected, fixed in 10% formalin, paraffin-embedded and cut in 5 µm thick sections. Immunohistochemical analysis was performed after antigen retrieval (5 min at 110°C in 0.1M citrate buffer pH6). The antibodies used in this study were anti-CD31 (1:200, ab28364, abcam), anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (1:1000, 9101S, Cell Signaling), anti-phospho-S6 ribosomal protein (Ser240/244) (1:1000, 2215S, Cell Signaling) and anti-phospho-4E-BP1 (Thr37/46) (236B4) (1:800, 2855S, Cell Signalling).

2.4 Results - *In vivo* CRISPR/Cas9-mediated gene mutation using MuLE vectors

Recent publications have illustrated the power of the CRISPR/Cas9 system for *in vivo* tumour engineering studies (241,250-252). Furthermore, we have demonstrated that CRISPR/Cas9 genome engineering is compatible with the MuLE system. A tetracistronic MuLE vector expressing sgRNA against *Cdkn2a*, expressing *H-Ras*^{G12V}, human Cas9 and puromycin resistance has been validated in *in vitro* tumour modelling studies (242). Additionally, we have shown that the combination of *H-Ras*^{G12V} and shRNA against *Cdkn2a* is sufficient to initiate angiosarcoma development *in vivo.* To test the potential of CRISPR/Cas9-mediated gene knockout for *in vivo* tumour engineering, 4-6-week-old SCID/beige mice were intravenously injected via the tail vein with concentrated ecotropic MuLE lentiviruses expressing sgRNA against *Cdkn2a,* expressing *hCas9*, *H-Ras*^{G12V} and a Luciferase reporter (Fig. 2.4.1A). The injection of this vector in the tail vein of male mice (n=2) induced increases in luciferase signals over 6 months (Fig. 2.4.1B). None of the injected female mice (n=3) showed any large increases in luciferase signal over time. Dissection of male mice revealed that the increased luciferase signals corresponded to the presence of bloody-appearing lesions in the testicles (Fig. 2.4.1C). Histological analysis of these lesions revealed a disrupted tissue architecture and malignant cells with atypical nuclei as well as large areas filled with red blood cells (Fig. 2.4.1D). To investigate whether the sgRNA induced efficient mutation of the *Cdkn2a* target gene, we performed a Surveyor assay (Fig. 2.4.1E). The abscence of cleaved products indicates no mutation of the targeted locus. Since we could not detect any cleaved product and the fact that only two out of five mice developed signals approximately 6 months after injection, it is likely that additional mutations have been acquired by the tumour-forming cells over time without the contribution of CRISPR/Cas9-mediated *Cdkn2a* knockout.

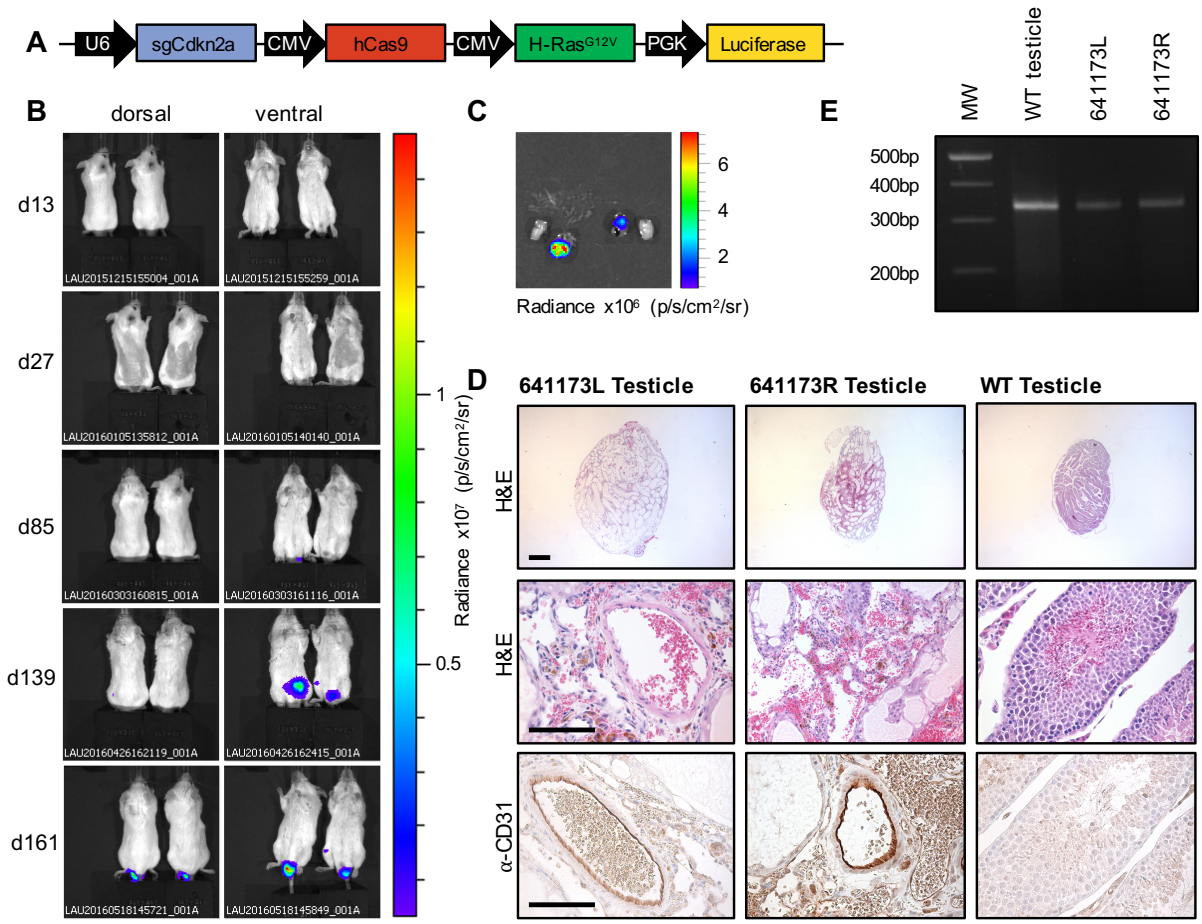


Figure 2.4.1. *In vivo* CRISPR/Cas9-mediated gene mutation using MuLE vectors. (**A**) Schematic of MuLE vector simultaneously expressing sgRNA against *Cdkn2a*, *hCas9*, *H-Ras^{G12V}* and a Luciferase reporter. (**B**) Bioluminescence imaging 13, 27, 85, 139 and 161 days after the injection of the afore-mentioned MuLE lentiviruse into the tail vein of 4-6-week-old SCID/beige. (**C**) Bioluminescence imaging of Lesion-bearing testicles. (**D**) Lesions showed large blood-filled spaces as well as a disrupted tissue architecture. Low magnification scale bar: 1000 µm and high magnification scale bar: 100 µm. (**E**) PCRs of the *Cdkn2a* locus targeted by the sgRNA were reannealed and subjected to Surveyor nuclease digestion. The abscence of cleaved products indicates no mutation of the targeted locus.

2.4.1 Methods - *In vivo* CRISPR/Cas9-mediated gene mutation using MuLE vectors

2.4.1.1 Surveyor nuclease assay

Genomic DNA was isolated from formalin-fixed paraffin-embedded tissues using the Arcturus® PicoPure® DNA extraction kit (KIT0103, ThermoFisher Scientific). PCRs for Surveyor assays were run using 20 ng genomic DNA, 0.5 µL 10mM dNTP mix (R0192, Fermentas), 2.5 µL 10x High Fidelity Buffer with 15mM $MgCl_2$, 0.4 µL High Fidelity Enzyme Mix (04738250001, Roche) and 0.75 µL mix of forward (Cdkn2a Exon 2 CTGATGATGATGGGCAACGT) and reverse primer (Cdkn2a Exon 2 reverse TGCTTGAGCTGAAGCTATGC) (10 mM) in a 25 µL reaction. 35 cycles of 94 °C 15sec, 55 °C 30 sec, 72 °C 45 sec were performed. The size of the amplified product was 334bp. 15 µL of the PCR product was used for formation of heteroduplexes and 7 µL of this was subjected to Surveyor nuclease digestion (Surveyor® Mutation Detection Kit, 706020, Integrated DNA Technologies) and analysed on a 1.5% (w/v) agarose gel in TAE buffer (40 mM Tris (pH 7.6), 20 mM acetic acid and 1 mM EDTA). BenchTop 100bp DNA Ladder (G8291, Promega) was used to estimate sizes.

3 DISCUSSION

3.1 Genetic background dictates whether H-Ras^{G12V} expression plus *Cdkn2a* knockdown causes angiosarcoma or undifferentiated pleomorphic sarcoma

The genomic characterization of soft tissue sarcomas revealed large numbers of genetic alterations in this cancer type. Although, this information is very useful for cancer research, there is still need for functional genetics to determine which of the alterations drive sarcomagenesis. The development of new *in vitro* and *in vivo* sarcoma models will help us to understand this complex process and the identification of driving mutations can increase our chances to develop more successful targeted therapies.

To examine sarcoma-relevant molecular pathways, we employed the MuLE lentiviral gene regulatory system (242) to attempt to generate *in vivo* models that reflect common molecular alterations of human angiosarcoma and UPS. The MuLE system allows the direct introduction of multiple genetic alterations in somatic cells *in vivo* by lentiviral injection. Guided by the genetics of human angiosarcomas, we functionally tested the contributions of the candidate sarcoma tumour suppressors *Cdkn2a*, *Trp53, Tsc2* and *Pten* and the candidate oncogenes *H-Ras, PIK3CA* and *c-Myc*. We discovered that the systemic injection of an ecotropic lentivirus expressing oncogenic *H-Ras* together with the knockdown of *Cdkn2a* or *Trp53* was sufficient to initiate angiosarcoma and/or UPS development. Unexpectedly, different mouse strains developed different types of sarcoma in response to identical genetic drivers, implicating genetic background as a major contributor to the genesis and spectrum of sarcomas.

Despite the fact that all mouse strains carry the same collection of genes, some factors such as allelic variants, sequence differences, epigenetic modifications and gene expression levels can influence a particular phenotype (253). Indeed, identical genetic alterations can result in different phenotypes when present in different mouse strain backgrounds. One example is the heterozygous *in vivo* inactivation of *Tsc1* in different mouse strains. Heterozygous *Tsc1* inactivation in BALB/c mice caused renal cell carcinoma development after 15-18 months (254), while it lead to post-natal death of unknown cause in C57BL/6 mice. Others showed that the incidence of mammary tumours varies among strains heterozygous for *Trp53*, with C57BL/6 mice being

resistant and BALB/c mice being susceptible (255,256). Both of these examples are due to the fact that BALB/c mice carry a rare allelic variant of *Cdkn2a* that leads to compromised p16 activity. This causes an increased susceptibility to develop certain tumours (257,258).

While angiosarcomas are thought to arise from endothelial cells, it is not clear whether UPS represent a group of de-differentiated sarcomas which share a common morphology but originated from different cell types or if all UPS tumours arise from a common cell of origin (40). It is rather unlikely that the tumours which developed in 129/Sv and C57BL/6 mice are de-differentiated angiosarcomas, because they all locate subcutaneously while none of the angiosarcomas which were found in 129/Sv, Fox Chase CB17 and SCID/beige mice were subcutaneous.

Adult mesenchymal stem cells (MSCs) have been proposed as the cell of origin of human UPS and bone marrow represents the main source of MSCs (42,259-261). It has been shown that the overexpression of *K-Ras* in cultured MSCs isolated from the bone marrow of *Trp53* knockout C57BL/6 mice lead to the transformation of these cells *in vitro*. Moreover, transformed, primary adult murine bone marrow-derived MSCs transplanted subcutaneously in nude mice, model UPS in these mice (262). We have shown that ecotropic MuLE lentiviruses can infect a variety of cultured primary cells, including mouse embryonic stem cells (242) and although bone marrow represents the main source of MSCs, peripheral blood-derived MSCs also exist in low numbers (263). Hence one hypothesis could be that we infected MSCs in the peripheral blood with the injected MuLE lentiviruses which then gave rise to UPS in 129/Sv and C57BL/6 mice.

Overall, our data demonstrate that the same combination of genetic drivers can cause different histological subtypes of soft tissue sarcomas. These observations highlight the importance of identifying the underlying molecular alterations of the tumour. As new therapies are developed, some will be applicable to any cancer, such as chemotherapeutic agents and radiotherapy, but in general it would be desirable to be able to treat tumours according to their underlying genetics rather than their histo-pathalogical appearance (12).

3.2 Characterisation of genetically-defined angiosarcoma cell lines

To complement the above-described *in vivo* studies with *in vitro* tumour modelling studies, we generated a panel of angiosarcoma cell lines expressing combinations of shRNA against *Cdkn2a* or shRNA-miR30 against *Trp53* with or without expression of *H-Ras*^{G12V}. Unexpectedly, wild type endothelial cells had the capacity to form blood-filled lesions in subcutaneous xenograft experiments.

It is known that the isolation of primary endothelial cells from their natural environment and propagation in cell culture can cause the loss of site-specific determinants on endothelial cell surfaces and thereby impair their physiological function (264). However, the fact that supposedly wild type endothelial cells provided by Cell Biologics possess tumour-initiating properties is alarming. It is known that primary endothelial cells that are maintained in culture for prolonged time can harbor aberrant chromosomes (265) and chromosomal instability can accelerate the accumulation of molecular alterations which might promote malignant transformation and tumour progression (266).

Based on the fact that wild type pMSECs showed tumour-initiating properties without the need of oncogene activation or tumour suppressor inactivation and the fact that most of the volume of the lesions was blood, which makes quantitative studies difficult, we decided to not continue with this alternative research strategy.

3.3 *In vivo* and *in vitro* testing of targeted therapies

Several clinical trials are testing therapeutic agents directed against signalling molecules that are frequently altered in human angiosarcomas. A significant disadvantage of these trials however, is that almost without exception, they are not being coupled to the analysis of the underlying molecular alterations in each individual patient. Therefore, we tested the therapeutic effects of a panel of 273 kinase inhibitors, which target a broad range of protein kinases, on the previously established *Cdkn2a* silenced and activated *H-Ras*-expressing endothelial cell line with the idea that we might be able to uncover therapeutic sensitivities that are dependent on the defined genetics of each cell line. The initial screen revealed that cells expressing oncogenic

H-Ras together with the knockdown of *Cdkn2a* were sensitive to mTOR/PI3K pathway inhibitors as well as CHK1 pathway inhibitors when compared to wild type cells. However, more detailed drug-response analyses revealed that there was no clear tendency for the cells with *H-Ras* and *Cdkn2a* gene alterations to be more sensitive to the inhibitors than those with wild type genes. One reason for this could be the slightly different experimental set up of the initial large drug screen, which was performed with the help of an automated liquid handling system, and the drug-response analysis, which was performed manually. While the automated system changed the media and the inhibitor every day for three days, the media plus inhibitor was only added once at the beginning of the manually performed drug-response analysis and then kept for three days. The observed differences in sensitivity between the automated screen and the manually performed drug-response analysis could be due to a different adherence of the cell lines. It is possible that sh*Cdkna* and *H-Ras*^{G12V} infected endothelial cells are less adherent than wild type cells and more prone to be lost during media change. Additional experiments would be needed to confirm this assumption.

We also tried to take advantage of our genetically-defined shRNA-*Cdkn2a* plus *H-Ras*^{G12V} driven angiosarcoma mouse model which incorporates some of the known driver mutations and pathways involved in sarcomagenesis, by asking whether this model could be used for the identification and testing of appropriate therapies specific for these combinatorial mutations. We decided to test two inhibitors targeting the MAPK and PI3K signalling pathways, namely the MEK inhibitor AZD6244 and the dual PI3K/mTOR inhibitor NVP-BEZ235. Unfortunately, no differences between vehicle and inhibitor-treated animals were observed. We concluded that these therapeutic regimes are not likely to be suitable for the treatment of Ras-driven human angiosarcomas.

One difficulty in targeting the PI3K/mTOR signalling pathway via kinase inhibition is that one tumour may express multiple receptors and growth factors, and PI3K may also be activated by MAPK signalling. Furthermore, the blockade of the PI3K downstream effector mTOR often leads to an unanticipated increase in PI3K and AKT activity (267). This feedback mechanism may be one reason for the poor response rates in cancer treatment to mTOR inhibitors. The largest phase II clinical study of mTOR inhibitors in metastatic and unresectable soft tissue sarcomas showed only a 2% response rate (268). Due to limited single-agent activity of PI3K, AKT and mTOR

at tolerated doses and to prevent unanticipated feedback loops, newer developed agents include dual PI3K and mTOR or MEK inhibitors (269). Up to date MEK and PI3K inhibitors have not yet shown significant clinical activity in Ras-driven cancers, for reasons related to feedback loops and poor therapeutic windows. Through a greater focus on the underlying genetic alterations and strategic application of rational combinations, it should be possible to maximize the potential of this promising new generation of targeted cancer agents.

MuLE vectors can carry an expression element for firefly luciferase in order to label infected cells and to trace tumour development *in vivo* over time via bioluminescence imaging. An issue with bioluminescence imaging is the wavelength-dependent transmission of light through animal tissues. There is an approximate tenfold loss of photon intensity for each centimeter of tissue depth. Moreover, images are surface weighted, which means that light sources closer to the surface of the animal appear brighter compared with deeper sources because of tissue attenuation properties (270). Since angiosarcomas in this model can arise anywhere in the body and due to significant mouse to mouse variability, the accurate temporal tracking of tumour progression and the quantitation of the tumour burden is difficult.

Overall our experiments show that the MuLE system can be used to establish a rapidly growing angiosarcoma mouse model that reflects the human disease. Furthermore, these studies provide proof-of-principle that the MuLE system can be used in the future for other genetic studies to systematically assess the contributions of other soft tissue sarcoma relevant genes to cellular transformation, tumour formation and progression. However, the accurate temporal tracking of tumour progression is difficult, which might be an obstacle for the screening of targeted therapies.

3.4 Limitations of the MuLE system

We have shown that the use of the MuLE system to combine different genetic elements represents a tool that facilitates systematic combinatorial genetic studies in mammalian cells. Furthermore, we have previously shown that ecotropic MuLE lentiviruses are able to transduce a wide variety of cell types *in vitro* and that we were able to establish *in vivo* models for three different tumour types, namely angiosarcoma,

UPS and high-grade endometrial stromal sarcoma (242). However, one real practical limitation of the MuLE system is that so far we were unable to provide proof that the MuLE system can not only be used to model sarcomas but also carcinomas *in vivo*.

Due to significant biosafety advantages, we decided to use lentiviruses pseudotyped with an ecotropic envelope protein from the Moloney murine leukemia virus (MMLV) (244). While ecotropic viruses can only infect rodent cells, amphotropic viral particles can infect most mammalian cells, including human cells. Their host range is determined primarily by the binding interaction between viral envelope surface glycoproteins and distinct proteins on the host cell surface that serve as viral receptors (271). In rodent cells a cationic amino acid transporter, termed CAT1, serves as the receptor for the envelope glycoprotein gp70 of ecotropic MMLV (272). CAT1 is expressed in epithelial cells in various tissues, including the endometrium. In the endometrium CAT1 is expressed on the basal surface of the epithelial cells (273). Limited accessibility of CAT1 could be one possible reason why we were not able to infect epithelial cells *in vivo*. Furthermore, others have observed that mucin, a glycoconjugate, inhibits the entry of lenti- and adenoviruses into epithelial cells. They identified that the inhibition of infection could be improved by pretreating either the cells or the virus with the glycolyse hydrolase neuraminidase before infection (274,275). It would be worth testing if the pre-treatment of ecotropic lentiviruses with neuraminidase increases epithelial cell infection *in vivo*.

Nonetheless, the experiments performed during this doctoral thesis demonstrate the genetic power of the MuLE system as well as its user friendliness, versatility and speed.

REFERENCES

1. Jemal A, Siegel R, Xu J, Ward E. Cancer Statistics, 2010. CA: A Cancer Journal for Clinicians. Wiley Subscription Services, Inc., A Wiley Company; 2010 Sep 1;60(5):277–300.

2. Stiller CA, Trama A, Serraino D, Rossi S, Navarro C, Chirlaque MD, et al. Descriptive epidemiology of sarcomas in Europe: report from the RARECARE project. Eur J Cancer. Elsevier; 2013 Feb;49(3):684–95.

3. Fletcher C. WHO classification of tumours of soft tissue and bone. Fourth Edition. 2013.

4. Lindor NM, McMaster ML, Lindor CJ. Concise handbook of familial cancer susceptibility syndromes—second edition. J Natl Cancer Inst. 2008.

5. Malkin D, Li FP, Strong LC, Fraumeni JF, Nelson CE, Kim DH, et al. Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. Science. American Association for the Advancement of Science; 1990 Nov 30;250(4985):1233–8.

6. Abramson DH, Ellsworth RM, Kitchin FD, Tung G. Second nonocular tumors in retinoblastoma survivors. Are they radiation-induced? Ophthalmology. Elsevier; 1984 Nov;91(11):1351–5.

7. King AA, Debaun MR, Riccardi VM, Gutmann DH. Malignant peripheral nerve sheath tumors in neurofibromatosis 1. American Journal of Medical Genetics. John Wiley & Sons, Inc; 2000 Aug 28;93(5):388–92.

8. Mark RJ, Poen J, Tran LM, Fu YS, Selch MT, Parker RG. Postirradiation sarcomas. A single-institution study and review of the literature. Cancer. 1994 May 15;73(10):2653–62.

9. Elliott P, Kleinschmidt I. Angiosarcoma of the liver in Great Britain in proximity to vinyl chloride sites. Occup Environ Med. BMJ Publishing Group Ltd; 1997 Jan 1;54(1):14–8.

10. Di Marco J, Kaci R, Orcel P, Nizard R, Laredo JD. Radiation-induced undifferentiated pleomorphic sarcoma after radiation therapy for a desmoid tumour. Cancer Radiother. 2016 Feb;20(1):36–8.

11. Seinen JM, Styring E, Verstappen V, Vult von Steyern F, Rydholm A, Suurmeijer AJH, et al. Radiation-associated angiosarcoma after breast cancer: high recurrence rate and poor survival despite surgical treatment with R0 resection. Ann Surg Oncol. 2012 Aug;19(8):2700–6.

12. Borden EC, Baker LH, Bell RS, Bramwell V, Demetri GD, Eisenberg BL, et al. Soft tissue sarcomas of adults: state of the translational science. Clin Cancer Res. American Association for Cancer Research; 2003 Jun;9(6):1941–56.

13. Helman LJ, Meltzer P. Mechanisms of sarcoma development. Nature Reviews Cancer. 2003 Sep;3(9):685–94.

14. Nielsen TO, West RB, Linn SC, Alter O, Knowling MA, O'Connell JX, et al. Molecular characterisation of soft tissue tumours: a gene expression study. The Lancet. 2002 Apr;359(9314):1301–7.

15. Turc-Carel C, Aurias A, Mugneret F, Lizard S, Sidaner I, Volk C, et al. Chromosomes in Ewing's sarcoma. I. An evaluation of 85 cases of remarkable consistency of t(11;22)(q24;q12). Cancer Genetics and Cytogenetics. Elsevier; 1988 Jun;32(2):229–38.

16. Bennicelli JL, Edwards RH, Barr FG. Mechanism for transcriptional gain of function resulting from chromosomal translocation in alveolar rhabdomyosarcoma. Proceedings of the National Academy of Sciences. National Academy of Sciences; 1996 May 28;93(11):5455–9.

17. Clark J, Rocques PJ, Crew AJ, Gill S, Shipley J, Chan AM, et al. Identification of novel genes, SYT and SSX, involved in the t(X;18)(p11.2;q11.2) translocation found in human synovial sarcoma. Nat Genet. Nature Publishing Group; 1994 Aug;7(4):502–8.

18. Mertens F, Antonescu CR, Hohenberger P, Ladanyi M, Modena P, D'Incalci M, et al. Translocation-Related Sarcomas. Seminars in Oncology. Elsevier; 2009 Aug;36(4):312–23.

19. Anderson JL, Denny CT, Tap WD, Federman N. Pediatric sarcomas: translating molecular pathogenesis of disease to novel therapeutic possibilities. Pediatric Research. Springer Nature; 2012;72(2):112–21.

20. Dei Tos AP, Maestro R, Doglioni C, Piccinin S, Libera DD, Boiocchi M, et al. Tumor suppressor genes and related molecules in leiomyosarcoma. The American Journal of Pathology. American Society for Investigative Pathology; 1996 Apr;148(4):1037–45.

21. Guillou L, Aurias A. Soft tissue sarcomas with complex genomic profiles. Virchows Arch. 2010 Feb;456(2):201–17.

22. Stratton MR, Moss S, Warren W, Patterson H, Clark J, Fisher C, et al. Mutation of the p53 gene in human soft tissue sarcomas: association with abnormalities of the RB1 gene. Oncogene. 1990 Sep;5(9):1297–301.

23. Pérot G, Chibon F, Montero A, Lagarde P, de Thé H, Terrier P, et al. Constant p53 Pathway Inactivation in a Large Series of Soft Tissue Sarcomas with Complex Genetics. The American Journal of Pathology. 2010 Oct;177(4):2080–90.

24. Cohen JA, Geradts J. Loss of RB and MTS1/CDKN2 (p16) expression in human sarcomas. Human Pathology. Elsevier; 1997 Aug;28(8):893–8.

25. Leach FS, Tokino T, Meltzer P, Burrell M, Oliner JD, Smith S, et al. p53 Mutation and MDM2 amplification in human soft tissue sarcomas. Cancer Res. American Association for Cancer Research; 1993 May 15;53(10 Suppl):2231–4.

26. Taylor BS, Barretina J, Maki RG, Antonescu CR, Singer S, Ladanyi M. Advances in sarcoma genomics and new therapeutic targets. Nature Reviews Cancer. Nature Publishing Group; 2011 Aug 1;11(8):541–57.

27. Barretina J, Taylor BS, Banerji S, Ramos AH, Lagos-Quintana M, Decarolis PL, et al. Subtype-specific genomic alterations define new targets for soft-tissue sarcoma therapy. Nat Genet. 2010 Aug;42(8):715–21.

28. Schöffski P, Cornillie J, Wozniak A, Li H, Hompes D. Soft tissue sarcoma: an update on systemic treatment options for patients with advanced disease. Oncol Res Treat. 2014;37(6):355–62.

29. Kauffman SL, Stout AP. Histiocytic tumors (fibrous xanthoma and histiocytoma) in children. Cancer. 1961 May;14:469–82.

30. Roholl PJM, Kleyne J, Elbers H, van der Vegt MCD, Albus-Lutter C, Van Unnik JAM. Characterization of tumour cells in malignant fibrous histiocytomas and other soft tissue tumours in comparison with malignant histiocytes. I. Immunohistochemical study on paraffin sections. The Journal of Pathology. John Wiley & Sons, Ltd; 1985 Oct;147(2):87–95.

31. Montgomery E, Fisher C. Myofibroblastic differentiation in malignant fibrous histiocytoma (pleomorphic myofibrosarcoma): a clinicopathological study. Histopathology. Blackwell Science Ltd; 2001 Jun;38(6):499–509.

32. Randall RL, Albritton KH, Ferney BJ, Layfield L. Malignant fibrous histiocytoma of soft tissue: an abandoned diagnosis. Am J Orthop (Belle Mead NJ). 2004 Dec;33(12):602–8.

33. Weiss SW, Enzinger FM. Malignant fibrous histiocytoma.An Analysis of 200 Cases. Cancer. Wiley Subscription Services, Inc., A Wiley Company; 1978 Jun;41(6):2250–66.

34. Gibbs JF, Huang PP, Lee RJ, McGrath B, Brooks J, McKinley B, et al. Malignant Fibrous Histiocytoma: An Institutional Reviews. Cancer Investigation. Taylor & Francis; 2001 Jan 30;19(1):23–7.

35. Le Doussal V, Coindre J-M, Leroux A, Hacene K, Terrier P, Bui NB, et al. Prognostic factors for patients with localized primary malignant fibrous histiocytoma: A multicenter study of 216 patients with multivariate analysis. Cancer. Wiley Subscription Services, Inc., A Wiley Company; 1996 May 1;77(9):1823–30.

36. Engellau J, Anderson H, Rydholm A, Bauer HCF, Hall KS, Gustafson P, et al. Time dependence of prognostic factors for patients with soft tissue sarcoma. Cancer. Wiley Subscription Services, Inc., A Wiley Company; 2004 May 15;100(10):2233–9.

37. Fletcher CDM. The evolving classification of soft tissue tumours - an update based on the new 2013 WHO classification. Histopathology. 2013 Oct 25;64(1):2–11.

38. Hollowood K, Fletcher CD. Malignant fibrous histiocytoma: morphologic pattern or pathologic entity? Semin Diagn Pathol. 1995 Aug;12(3):210–20.

39. Castro-Malaspina H, Gay RE, Resnick G, Kapoor N, Meyers P, Chiarieri D, et al. Characterization of human bone marrow fibroblast colony-forming cells (CFU-F) and their progeny. Blood. 1980 Aug;56(2):289–301.

40. Matushansky I, Charytonowicz E, Mills J, Siddiqi S, Hricik T, Cordon-Cardo C. MFH classification: differentiating undifferentiated pleomorphic sarcoma in the 21st Century. Expert Rev Anticancer Ther. 2009 Aug;9(8):1135–44.

41. Barry Pierce G, Johnson LD. Differentiation and cancer. In Vitro. 1971 Nov;7(3):140–5.

42. Mohseny AB, Hogendoorn PCW. Concise review: mesenchymal tumors: when stem cells go mad. Stem Cells. Wiley Subscription Services, Inc., A Wiley Company; 2011 Mar;29(3):397–403.

43. Gazziola C, Cordani N, Wasserman B, Carta S, Colombatti A, Perris R. Malignant fibrous histiocytoma: a proposed cellular origin and identification of its characterizing gene transcripts. Int J Oncol. Spandidos Publications; 2003 Aug 1;23(2):343–51.

44. Matushansky I, Hernando E, Socci ND, Mills JE, Matos TA, Edgar MA, et al. Derivation of sarcomas from mesenchymal stem cells via inactivation of the Wnt pathway. J Clin Invest. American Society for Clinical Investigation; 2007 Nov 1;117(11):3248–57.

45. Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, et al. Integrative Analysis of Complex Cancer Genomics and Clinical Profiles Using the cBioPortal. Science signaling. NIH Public Access; 2013 Apr 2;6(269):pl1–pl1.

46. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov. American Association for Cancer Research; 2012 May;2(5):401–4.

47. Hernando E, Charytonowicz E, Dudas ME, Menendez S, Matushansky I, Mills J, et al. The AKT-mTOR pathway plays a critical role in the development of leiomyosarcomas. Nature Medicine. 2007 May 13;13(6):748–53.

48. Yoo J, Robinson RA, Lee JY. H-ras and K-ras gene mutations in primary human soft tissue sarcoma: concomitant mutations of the ras genes. Mod Pathol. 1999 Aug;12(8):775–80.

49. Yoo J, Robinson RA. H-ras and K-ras mutations in soft tissue sarcoma. Cancer. John Wiley & Sons, Inc; 1999 Jul 1;86(1):58–63.

50. Li B, Li L, Li X, Wang Y, Xie Y, Liu C, et al. Undifferentiated pleomorphic sarcoma with co-existence of KRAS/PIK3CA mutations. Int J Clin Exp Pathol. e-Century Publishing Corporation; 2015;8(7):8563–7.

51. Weiss SW, Goldblum JR. Malignant vascular tumors. Enzinger and Weiss's soft tissue tumors; 2001.

52. Young RJ, Brown NJ, Reed MW, Hughes D, Woll PJ. Angiosarcoma. The Lancet Oncology. 2010 Oct;11(10):983–91.

53. Mark RJ, Poen JC, Tran LM, Fu YS, Juillard GF. Angiosarcoma. A report of 67 patients and a review of the literature. Cancer. Wiley Subscription Services, Inc., A Wiley Company; 1996 Jun 1;77(11):2400–6.

54. Espat NJ, Lewis JJ, Woodruff JM, Antonescu C. Confirmed angiosarcoma: prognostic factors and outcome in 50 prospectively followed patients. …. 2000.

55. Coindre J-M, Terrier P, Guillou L, Le Doussal V, Collin F, Ranchère D, et al. Predictive value of grade for metastasis development in the main histologic types of adult soft tissue sarcomas. Cancer. John Wiley & Sons, Inc; 2001 May 15;91(10):1914–26.

56. Antonescu C. Malignant vascular tumors--an update. Mod Pathol. Nature Publishing Group; 2014 Jan;27 Suppl 1:S30–8.

57. Jesse Hart, Srinivas Mandavilli. Epithelioid Angiosarcoma: A Brief Diagnostic Review and Differential Diagnosis. College of American Pathologist 325 Waukegan Rd, Northfield, IL 60093; 2011 Feb 1.

58. Miettinen M, Fetsch JF. Distribution of keratins in normal endothelial cells and a spectrum of vascular tumors: implications in tumor diagnosis. Human Pathology. Elsevier; 2000 Sep;31(9):1062–7.

59. Miettinen M, Lindenmayer AE, Chaubal A. Endothelial cell markers CD31, CD34, and BNH9 antibody to H- and Y-antigens--evaluation of their specificity and sensitivity in the diagnosis of vascular tumors and comparison with von Willebrand factor. Mod Pathol. 1994 Jan;7(1):82–90.

60. Folpe AL, Chand EM, Goldblum JR, Weiss SW. Expression of Fli-1, a nuclear transcription factor, distinguishes vascular neoplasms from potential mimics. The American Journal of Surgical Pathology. 2001 Aug;25(8):1061–6.

61. DeYoung BR, Swanson PE, Argenyi ZB, Ritter JH, Fitzgibbon JF, Stahl DJ, et al. CD31 immunoreactivity in mesenchymal neoplasms of the skin and subcutis: report of 145 cases and review of putative immunohistologic markers of endothelial differentiation. J Cutan Pathol. 1995 Jun;22(3):215–22.

62. Stewart FW, Treves N. Lymphangiosarcoma in Postmastectomy Lymphedema: A Report of Six Cases in Elephantiasis Chirurgica. CA: A Cancer Journal for Clinicians. John Wiley & Sons, Ltd; 1981 Sep 1;31(5):284–99.

63. Virtanen A, Pukkala E, Auvinen A. Angiosarcoma after radiotherapy: a cohort study of 332 163 Finnish cancer patients. British Journal of Cancer. Nature Publishing Group; 2007 May 22;97(1):115–7.

64. Bosetti C, La Vecchia C, Lipworth L, McLaughlin JK. Occupational exposure to vinyl chloride and cancer risk: a review of the epidemiologic literature. European Journal of Cancer Prevention. Find this article online; 2003 Oct;12(5):427–30.

65. Ron E. Cancer risks from medical radiation. Health Phys. 2003 Jul;85(1):47–59.

66. Locker GY, Doroshow JH, Zwelling LA, Chabner BA. The clinical features of hepatic angiosarcoma: a report of four cases and a review of the English literature. Medicine (Baltimore). 1979 Jan;58(1):48–64.

67. Zietz C, Rössle M, Haas C, Sendelhofert A, Hirschmann A, Stürzl M, et al. MDM-2 Oncoprotein Overexpression, p53 Gene Mutation, and VEGF Up-Regulation in Angiosarcomas. The American Journal of Pathology. 1998 Nov;153(5):1425–33.

68. Manner J, Radlwimmer B, Hohenberger P, Mössinger K, Küffer S, Sauer C, et al. MYC High Level Gene Amplification Is a Distinctive Feature of Angiosarcomas after Irradiation or Chronic Lymphedema. The American Journal of Pathology. 2010 Jan;176(1):34–9.

69. Weihrauch M, Bader M, Lehnert G, Koch B, Wittekind C, Wrbitzky R, et al. Mutation analysis of K-ras-2 in liver angiosarcoma and adjacent nonneoplastic liver tissue from patients occupationally exposed to vinyl chloride. Environmental and Molecular Mutagenesis. Wiley Subscription Services, Inc., A Wiley Company; 2002 Jan 1;40(1):36–40.

70. Hayashi T, Koike K, Kumasaka T, Saito T, Mitani K, Terao Y, et al. Uterine angiosarcoma associated with lymphangioleiomyomatosis in a patient with tuberous sclerosis complex: an autopsy case report with immunohistochemical and genetic analysis. Human Pathology. 2012 Oct;43(10):1777–84.

71. Guo T, Zhang L, Chang N-E, Singer S, Maki RG, Antonescu CR. Consistent MYC and FLT4 gene amplification in radiation-induced angiosarcoma but not in other radiation-associated atypical vascular lesions. Genes Chromosomes Cancer. Wiley Subscription Services, Inc., A Wiley Company; 2011 Jan;50(1):25–33.

72. Behjati S, Tarpey PS, Sheldon H, Martincorena I, Van Loo P, Gundem G, et al. Recurrent PTPRB and PLCG1 mutations in angiosarcoma. Nat Genet. Nature Research; 2014 Apr 1;46(4):376–9.

73. Murali R, Chandramohan R, Möller I, Scholz SL, Berger M, Huberman K, et al. Targeted massively parallel sequencing of angiosarcomas reveals frequent activation of the mitogen activated protein kinase pathway. Oncotarget. Impact Journals, LLC; 2015 Nov 3;6(34):36041–52.

74. Italiano A, Chen C-L, Thomas R, Breen M, Bonnet F, Sevenet N, et al. Alterations of the p53 and PIK3CA/AKT/mTOR pathways in angiosarcomas: a pattern distinct from other sarcomas with complex genomics. Cancer. Wiley Subscription Services, Inc., A Wiley Company; 2012 Dec 1;118(23):5878–87.

75. Tate G, Suzuki T, Mitsuya T. Mutation of the PTEN gene in a human hepatic angiosarcoma. Cancer Genetics and Cytogenetics. 2007 Oct 15;178(2):160–2.

76. Folkman J. Angiogenesis. Annual Review of Medicine. Annual Reviews; 2006 Jan 12;57(1):1–18.

77. Arbiser JL, Moses MA, Fernandez CA, Ghiso N, Cao Y, Klauber N, et al. Oncogenic H-ras stimulates tumor angiogenesis by two distinct pathways. Proceedings of the National Academy of Sciences. National Acad Sciences; 1997 Feb 4;94(3):861–6.

78. Shweiki D, Itin A, Soffer D, Keshet E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature; 1992.

79. Baeriswyl V, Christofori G. The angiogenic switch in carcinogenesis. Semin Cancer Biol. 2009 Oct;19(5):329–37.

80. Pugh CW, Ratcliffe PJ. Regulation of angiogenesis by hypoxia: role of the HIF system. Nature Medicine. Nature Publishing Group; 2003 Jun 1;9(6):677–84.

81. Bergers G, Benjamin LE. Angiogenesis: Tumorigenesis and the angiogenic switch. Nature Reviews Cancer. 2003 Jun;3(6):401–10.

82. Shibuya M. Vascular endothelial growth factor-dependent and -independent regulation of angiogenesis. BMB Reports. 2008 Apr 30;41(4):278–86.

83. Takeda A, Stoeltzing O, Ahmad SA, Reinmuth N, Liu W, Parikh A, et al. Role of angiogenesis in the development and growth of liver metastasis. Ann Surg Oncol. 2002 Aug;9(7):610–6.

84. Ferrara N. Vascular Endothelial Growth Factor. Arterioscler Thromb Vasc Biol. American Heart Association, Inc; 2009 Jun 1;29(6):789–91.

85. Mac Gabhann F, Popel AS. Systems Biology of Vascular Endothelial Growth Factors. Microcirculation. Taylor & Francis; 2009 Jul 10.

86. Carmeliet P. VEGF as a Key Mediator of Angiogenesis in Cancer. Oncology. Karger Publishers; 2005 Nov 23;69(Suppl. 3):4–10.

87. Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, et al. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature. Nature Publishing Group; 1996 Apr 4;380(6573):435–9.

88. Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, et al. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature. Nature Publishing Group; 1996 Apr 4;380(6573):439–42.

89. Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, et al. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature. Nature Publishing Group; 1995 Jul 6;376(6535):62–6.

90. Olsson A-K, Dimberg A, Kreuger J, Claesson-Welsh L. VEGF receptor signalling — in control of vascular function. Nature Reviews Molecular Cell Biology. 2006 May;7(5):359–71.

91. Ferrara N, Gerber H-P, LeCouter J. The biology of VEGF and its receptors. Nature Medicine. Nature Publishing Group; 2003 Jun 1;9(6):669–76.

92. Bäumer S, Keller L, Holtmann A, Funke R, August B, Gamp A, et al. Vascular endothelial cell-specific phosphotyrosine phosphatase (VE-PTP) activity is required for blood vessel development. Blood. American Society of Hematology; 2006 Jun 15;107(12):4754–62.

93. Hayashi M, Majumdar A, Li X, Adler J, Sun Z, Vertuani S, et al. VE-PTP regulates VEGFR2 activity in stalk cells to establish endothelial cell polarity and lumen formation. Nat Commun. Nature Publishing Group; 2013 Apr 9;4:1672.

94. Mellberg S, Dimberg A, Bahram F, Hayashi M, Rennel E, Ameur A, et al. Transcriptional profiling reveals a critical role for tyrosine phosphatase VE-PTP in regulation of VEGFR2 activity and endothelial cell morphogenesis. FASEB J. Federation of American Societies for Experimental Biology; 2009 May;23(5):1490–502.

95. Dominguez MG, Hughes VC, Pan L, Simmons M, Daly C, Anderson K, et al. Vascular endothelial tyrosine phosphatase (VE-PTP)-null mice undergo vasculogenesis but die embryonically because of defects in angiogenesis. Proceedings of the National Academy of Sciences. National Acad Sciences; 2007 Feb 27;104(9):3243–8.

96. Yonemori K, Tsuta K, Ando M, Hirakawa A, Hatanaka Y, Matsuno Y, et al. Contrasting Prognostic Implications of Platelet-Derived Growth Factor Receptor-β and Vascular Endothelial Growth Factor Receptor-2 in Patients with Angiosarcoma. Ann Surg Oncol. Springer-Verlag; 2011;18(10):2841–50.

97. Itakura E, Yamamoto H, Oda Y, Tsuneyoshi M. Detection and characterization of vascular endothelial growth factors and their receptors in a series of angiosarcomas. Journal of Surgical Oncology. Wiley Subscription Services, Inc., A Wiley Company; 2008 Jan 1;97(1):74–81.

98. Antonescu CR, Yoshida A, Guo T, Chang NE, Zhang L, Agaram NP, et al. KDR Activating Mutations in Human Angiosarcomas Are Sensitive to Specific Kinase Inhibitors. Cancer Res. American Association for Cancer Research; 2009 Sep 14;69(18):7175–9.

99. Arbiser JL, Larsson H, Claesson-Welsh L, Bai X, LaMontagne K, Weiss SW, et al. Overexpression of VEGF 121 in immortalized endothelial cells causes conversion to slowly growing angiosarcoma and high level expression of the VEGF receptors VEGFR-1 and VEGFR-2 in vivo. The American Journal of Pathology. 2000 Apr;156(4):1469–76.

100. Ghahremani MF, Radaelli E, Haigh K, Bartunkova S, Haenebalcke L, Marine J-C, et al. Loss of autocrine endothelial-derived VEGF significantly reduces hemangiosarcoma development in conditional p53-deficient mice. Cell Cycle. Taylor & Francis; 2014 Mar 11;13(9):1501–7.

101. Naka N, Tomita Y, Nakanishi H, Araki N, Hongyo T, Ochi T, et al. Mutations of p53 tumor-suppressor gene in angiosarcoma. International Journal of Cancer. Wiley Subscription Services, Inc., A Wiley Company; 1997 Jun 11;71(6):952–5.

102. Domfeh AB, Fichera M, Hunt JL. Allelic loss of 3 different tumor suppressor gene loci in benign and malignant endothelial tumors of the head and neck. Arch Pathol Lab Med. 2006 Aug;130(8):1184–7.

103. Zu Y, Perle MA, Yan Z, Liu J, Kumar A, Waisman J. Chromosomal abnormalities and p53 gene mutation in a cardiac angiosarcoma. Appl Immunohistochem Mol Morphol. 2001 Mar;9(1):24–8.

104. Amo Y, Masuzawa M, Hamada Y, Takasu H, Fujimura T, Katsuoka K, et al. Serum levels of vascular endothelial growth factor in a haemangiosarcoma patient with a newly typed p53 gene point mutation. Br J Dermatol. 2000 Nov;143(5):1118–9.

105. Garcia JM, Gonzalez R, Silva JM, Dominguez G, Vegazo IS, Gamallo C, et al. Mutational status of K-ras and TP53 genes in primary sarcomas of the heart. British Journal of Cancer. Nature Publishing Group; 2000 Mar;82(6):1183–5.

106. Soini Y, Welsh JA, Ishak KG, Bennett WP. p53 mutations in primary hepatic angiosarcomas not associated with vinyl chloride exposure. Carcinogenesis. 1995 Nov;16(11):2879–81.

107. Przygodzki RM, Finkelstein SD, Keohavong P, Zhu D, Bakker A, Swalsky PA, et al. Sporadic and Thorotrast-induced angiosarcomas of the liver manifest frequent and multiple point mutations in K-ras-2. Lab Invest. 1997 Jan;76(1):153–9.

108. Tannapfel A, Weihrauch M, Benicke M, Uhlmann D, Hauss J, Wrbitzky R, et al. p16INK4A – alterations in primary angiosarcoma of the liver. Journal of Hepatology. 2001 Jul;35(1):62–7.

109. Zilfou JT, Lowe SW. Tumor suppressive functions of p53. Cold Spring Harb Perspect Biol. Cold Spring Harbor Lab; 2009 Nov;1(5):a001883–3.

110. Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CA, Butel JS, et al. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature. Nature Publishing Group; 1992 Mar 19;356(6366):215–21.

111. Akashi M, Koeffler HP. Li-Fraumeni syndrome and the role of the p53 tumor suppressor gene in cancer susceptibility. Clin Obstet Gynecol. 1998 Mar;41(1):172–99.

112. Hainaut P, Hollstein M. p53 and human cancer: the first ten thousand mutations. Adv Cancer Res. 2000;77:81–137.

113. Sherr CJ, Weber JD. The ARF/p53 pathway. Current Opinion in Genetics & Development. 2000 Feb;10(1):94–9.

114. Zhang Y, Xiong Y, Yarbrough WG. ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. Cell. Elsevier; 1998 Mar 20;92(6):725–34.

115. Bieging KT, Mello SS, Attardi LD. Unravelling mechanisms of p53-mediated tumour suppression. Nature Reviews Cancer. Nature Research; 2014 May 1;14(5):359–70.

116. Horn HF, Vousden KH. Coping with stress: multiple ways to activate p53. Oncogene. Nature Publishing Group; 2007 Feb 26;26(9):1306–16.

117. Yee KS, Vousden KH. Complicating the complexity of p53. Carcinogenesis. Oxford University Press; 2005 Aug;26(8):1317–22.

118. Kubbutat MH, Jones SN, Vousden KH. Regulation of p53 stability by Mdm2. Nature. 1997 May 15;387(6630):299–303.

119. Haupt Y, Maya R, Kazaz A, Oren M. Mdm2 promotes the rapid degradation of p53. Nature. 1997 May 15;387(6630):296–9.

120. Michael D, Oren M. The p53-Mdm2 module and the ubiquitin system. Semin Cancer Biol. 2003 Feb;13(1):49–58.

121. Momand J, Wu HH, Dasgupta G. MDM2--master regulator of the p53 tumor suppressor protein. Gene. 2000 Jan 25;242(1-2):15–29.

122. Sharpless NE, DePinho RA. The INK4A/ARF locus and its two gene products. Current Opinion in Genetics & Development. 1999 Feb;9(1):22–30.

123. Kamijo T, Bodner S, van de Kamp E, Randle DH, Sherr CJ. Tumor spectrum in ARF-deficient mice. Cancer Res. 1999 May 1;59(9):2217–22.

124. Kamijo T, Zindy F, Roussel MF, Quelle DE, Downing JR, Ashmun RA, et al. Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF. Cell. 1997 Nov 28;91(5):649–59.

125. Sharpless NE, Bardeesy N, Lee KH, Carrasco D, Castrillon DH, Aguirre AJ, et al. Loss of p16Ink4a with retention of p19Arf predisposes mice to tumorigenesis. Nature. Nature Publishing Group; 2001 Sep 6;413(6851):86–91.

126. Serrano M, Lee H, Chin L, Cordon-Cardo C, Beach D, DePinho RA. Role of the INK4a locus in tumor suppression and cell mortality. Cell. 1996 Apr 5;85(1):27–37.

127. Sherr CJ. Ink4-Arf locus in cancer and aging. Wiley Interdiscip Rev Dev Biol. John Wiley & Sons, Inc; 2012 Sep;1(5):731–41.

128. Sherr CJ. The INK4a/ARF network in tumour suppression. Nature Reviews Molecular Cell Biology. 2001 Oct;2(10):731–7.

129. Verbeke SLJ, Bertoni F, Bacchini P, Oosting J, Sciot R, Krenács T, et al. Active TGF-β signaling and decreased expression of PTEN separates angiosarcoma of bone from its soft tissue counterpart. Mod Pathol. Nature Publishing Group; 2013 Sep;26(9):1211–21.

130. Barbin A, Froment O, Boivin S, Marion MJ, Belpoggi F, Maltoni C, et al. p53 gene mutation pattern in rat liver tumors induced by vinyl chloride. Cancer Res. American Association for Cancer Research; 1997 May 1;57(9):1695–8.

131. Pylayeva-Gupta Y, Grabocka E, Bar-Sagi D. RAS oncogenes: weaving a tumorigenic web. Nature Reviews Cancer. Nature Publishing Group; 2011 Oct 13;11(11):761–74.

132. Karnoub AE, Weinberg RA. Ras oncogenes: split personalities. Nature Reviews Molecular Cell Biology. Nature Publishing Group; 2008 Jul;9(7):517–31.

133. Prior IA, Lewis PD, Mattos C. A comprehensive survey of Ras mutations in cancer. Cancer Res. American Association for Cancer Research; 2012 May 15;72(10):2457–67.

134. Quinlan MP, Settleman J. Isoform-specific ras functions in development and cancer. Future Oncol. Future Medicine London, UK; 2009 Feb;5(1):105–16.

135. Johnson L, Greenbaum D, Cichowski K, Mercer K, Murphy E, Schmitt E, et al. K-ras is an essential gene in the mouse with partial functional overlap with N-ras. Genes Dev. Cold Spring Harbor Laboratory Press; 1997 Oct 1;11(19):2468–81.

136. Leon J, Guerrero I, Pellicer A. Differential expression of the ras gene family in mice. Molecular and Cellular Biology. American Society for Microbiology; 1987 Apr;7(4):1535–40.

137. Newbold RF, Overell RW. Fibroblast immortality is a prerequisite for transformation by EJ c-Ha-ras oncogene. Nature. Nature Publishing Group; 1983 Aug 18;304(5927):648–51.

138. Land H, Parada LF, Weinberg RA. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature. Nature Publishing Group; 1983 Aug 18;304(5927):596–602.

139. Ruley HE. Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. Nature. Nature Publishing Group; 1983 Aug 18;304(5927):602–6.

140. Bos JL, Rehmann H, Wittinghofer A. GEFs and GAPs: Critical Elements in the Control of Small G Proteins. Cell. Elsevier; 2007 Jul;130(2):385.

141. Clark R, Wong G, Arnheim N, Nitecki D, McCormick F. Antibodies specific for amino acid 12 of the ras oncogene product inhibit GTP binding. Proceedings of the National Academy of Sciences. National Acad Sciences; 1985 Aug;82(16):5280–4.

142. Cargnello M, Roux PP. Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. Microbiol Mol Biol Rev. American Society for Microbiology; 2011 Mar;75(1):50–83.

143. Rajagopalan H, Bardelli A, Lengauer C, Kinzler KW, Vogelstein B, Velculescu VE. Tumorigenesis: RAF/RAS oncogenes and mismatch-repair status. Nature. Nature Publishing Group; 2002 Aug 29;418(6901):934–4.

144. Bajaj A, Zheng Q, Adam A, Vincent P, Pumiglia K. Activation of Endothelial Ras Signaling Bypasses Senescence and Causes Abnormal Vascular Morphogenesis. Cancer Res. American Association for Cancer Research; 2010 Apr 28;70(9):3803–12.

145. Hemmings BA, Restuccia DF. The PI3K-PKB/Akt pathway. Cold Spring Harb Perspect Biol. Cold Spring Harbor Lab; 2015 Apr 1;7(4):a026609.

146. Fruman DA, Rommel C. PI3K and cancer: lessons, challenges and opportunities. Nature Reviews Drug Discovery. 2014 Jan 31;13(2):140–56.

147. Samuels Y, Ericson K. Oncogenic PI3K and its role in cancer. Current Opinion in Oncology. 2006 Jan;18(1):77–82.

148. Song MS, Salmena L, Pandolfi PP. The functions and regulation of the PTEN tumour suppressor. Nature Reviews Molecular Cell Biology. 2012 Apr 4.

149. Engelman JA. Targeting PI3K signalling in cancer: opportunities, challenges and limitations. Nature Reviews Cancer. 2009 Aug;9(8):550–62.

150. Vanhaesebroeck B, Guillermet-Guibert J, Graupera M, Bilanges B. The emerging mechanisms of isoform-specific PI3K signalling. Nature Reviews Molecular Cell Biology. 2010 Apr 9;11(5):329–41.

151. Castellano E, Downward J. RAS Interaction with PI3K: More Than Just Another Effector Pathway. Genes Cancer. SAGE Publications; 2011 Mar;2(3):261–74.

152. Vogt PK, Kang S, Elsliger M-A, Gymnopoulos M. Cancer-specific mutations in phosphatidylinositol 3-kinase. Trends in Biochemical Sciences. Elsevier; 2007 Jul;32(7):342–9.

153. Shioi T, Kang PM, Douglas PS, Hampe J, Yballe CM, Lawitts J, et al. The conserved phosphoinositide 3-kinase pathway determines heart size in mice. EMBO J. EMBO Press; 2000 Jun 1;19(11):2537–48.

154. Adams JR, Xu K, Liu JC, Agamez NMR, Loch AJ, Wong RG, et al. Cooperation between Pik3ca and p53 mutations in mouse mammary tumor formation. Cancer Res. American Association for Cancer Research; 2011 Apr 1;71(7):2706–17.

155. Di Cristofano A, Pesce B, Cordon-Cardo C, Pandolfi PP. Pten is essential for embryonic development and tumour suppression. Nat Genet. 1998 Aug;19(4):348–55.

156. Suzuki A, la Pompa de JL, Stambolic V, Elia AJ, Sasaki T, Barrantes IDB, et al. High cancer susceptibility and embryonic lethality associated with mutation of the PTEN tumor suppressor gene in mice. Current Biology. 1998 Oct;8(21):1169–78.

157. Stambolic V, Tsao M-S, Macpherson D, Suzuki A, Chapman WB, Mak TW. High Incidence of Breast and Endometrial Neoplasia Resembling Human Cowden Syndrome in pten+/− Mice. Cancer Res. American Association for Cancer Research; 2000 Jul 1;60(13):3605–11.

158. Chow LML, Baker SJ. PTEN function in normal and neoplastic growth. Cancer Letters. Elsevier; 2006 Sep 28;241(2):184–96.

159. Laplante M, Sabatini DM. mTOR Signaling in Growth Control and Disease. Cell. 2012 Apr;149(2):274–93.

160. Manning BD, Cantley LC. AKT/PKB Signaling: Navigating Downstream. Cell. 2007 Jun;129(7):1261–74.

161. Inoki K, Li Y, Zhu T, Wu J, Guan K-L. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat Cell Biol. Nature Publishing Group; 2002 Sep;4(9):648–57.

162. Huang J, Manning BD. The TSC1-TSC2 complex: a molecular switchboard controlling cell growth. Biochem J. Portland Press Limited; 2008 Jun 1;412(2):179–90.

163. Young J, Povey S. The genetic basis of tuberous sclerosis. Molecular Medicine Today. Elsevier; 1998 Jul;4(7):313–9.

164. Onda H, Lueck A, Marks PW, Warren HB, Kwiatkowski DJ. Tsc2+/– mice develop tumors in multiple sites that express gelsolin and are influenced by genetic background. J Clin Invest. American Society for Clinical Investigation; 1999 Sep 15;104(6):687–95.

165. Kobayashi T, Minowa O, Kuno J, Mitani H, Hino O, Noda T. Renal Carcinogenesis, Hepatic Hemangiomatosis, and Embryonic Lethality Caused by a Germ-Line Tsc2 Mutation in Mice. Cancer Res. American Association for Cancer Research; 1999 Mar 15;59(6):1206–11.

166. Perry B, Banyard J, McLaughlin ER, Watnick R, Sohn A, Brindley DN, et al. AKT1 overexpression in endothelial cells leads to the development of cutaneous vascular malformations in vivo. Arch Dermatol. American Medical Association; 2007 Apr;143(4):504–6.

167. Davis AC, Wims M, Spotts GD, Hann SR, Bradley A. A null c-myc mutation causes lethality before 10.5 days of gestation in homozygotes and reduced fertility in heterozygous female mice. Genes Dev. Cold Spring Harbor Lab; 1993 Apr;7(4):671–82.

168. Morton JP, Sansom OJ. MYC-y mice: from tumour initiation to therapeutic targeting of endogenous MYC. Mol Oncol. 2013 Apr;7(2):248–58.

169. Dang CV. MYC on the path to cancer. Cell. 2012 Mar 30;149(1):22–35.

170. Zack TI, Schumacher SE, Carter SL, Cherniack AD, Saksena G, Tabak B, et al. Pan-cancer patterns of somatic copy number alteration. Nat Genet. Nature Research; 2013 Oct 1;45(10):1134–40.

171. Klein G. Specific chromosomal translocations and the genesis of B-cell-derived tumors in mice and men. Cell. Cell Press; 1983 Feb;32(2):311–5.

172. Pomerantz MM, Ahmadiyeh N, Jia L, Herman P, Verzi MP, Doddapaneni H, et al. The 8q24 cancer risk variant rs6983267 shows long-range interaction with MYC in colorectal cancer. Nat Genet. Nature Publishing Group; 2009 Aug;41(8):882–4.

173. Wright JB, Brown SJ, Cole MD. Upregulation of c-MYC in cis through a large chromatin loop linked to a cancer risk-associated single-nucleotide polymorphism in colorectal cancer cells. Molecular and Cellular Biology. American Society for Microbiology; 2010 Mar;30(6):1411–20.

174. He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, et al. Identification of c-MYC as a target of the APC pathway. Science. 1998 Sep 4;281(5382):1509–12.

175. Palomero T, Lim WK, Odom DT, Sulis ML, Real PJ, Margolin A, et al. NOTCH1 directly regulates c-MYC and activates a feed-forward-loop transcriptional network promoting leukemic cell growth. Proceedings of the National Academy of Sciences. National Acad Sciences; 2006 Nov 28;103(48):18261–6.

176. Blackwood EM, Eisenman RN. Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc. Science. 1991 Mar 8;251(4998):1211–7.

177. Ginter PS, Mosquera JM, MacDonald TY, D'Alfonso TM, Rubin MA, Shin SJ. Diagnostic utility of MYC amplification and anti-MYC immunohistochemistry in atypical vascular lesions, primary or radiation-induced mammary angiosarcomas, and primary angiosarcomas of other sites. Human Pathology. 2014 Apr;45(4):709–16.

178. Mentzel T, Schildhaus HU, Palmedo G, Büttner R, Kutzner H. Postradiation cutaneous angiosarcoma after treatment of breast carcinoma is characterized by MYC amplification in contrast to atypical vascular lesions after radiotherapy and control cases: clinicopathological, immunohistochemical and molecular analysis of 66 cases. Mod Pathol. Nature Publishing Group; 2012 Jan;25(1):75–85.

179. Laé M, Lebel A, Hamel-Viard F, Asselain B, Trassard M, Sastre X, et al. Can c-myc amplification reliably discriminate postradiation from primary angiosarcoma of the breast? Cancer Radiother. 2015 May;19(3):168–74.

180. Shon W, Sukov WR, Jenkins SM, Folpe AL. MYC amplification and overexpression in primary cutaneous angiosarcoma: a fluorescence in-situ hybridization and immunohistochemical study. Mod Pathol. Nature Publishing Group; 2014 Apr;27(4):509–15.

181. Udager AM, Ishikawa MK, Lucas DR, McHugh JB, Patel RM. MYC immunohistochemistry in angiosarcoma and atypical vascular lesions: practical considerations based on a single institutional experience. Pathology. 2016 Dec;48(7):697–704.

182. Kandoth C, McLellan MD, Vandin F, Ye K, Niu B, Lu C, et al. Mutational landscape and significance across 12 major cancer types. Nature. Nature Research; 2013 Oct 17;502(7471):333–9.

183. Ciriello G, Miller ML, Aksoy BA, Senbabaoglu Y, Schultz N, Sander C. Emerging landscape of oncogenic signatures across human cancers. Nat Genet. Nature Research; 2013 Oct 1;45(10):1127–33.

184. Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz LA, Kinzler KW. Cancer Genome Landscapes. Science. American Association for the Advancement of Science; 2013 Mar 29;339(6127):1546–58.

185. Cohen SM, Storer RD, Criswell KA, Doerrer NG, Dellarco VL, Pegg DG, et al. Hemangiosarcoma in Rodents: Mode-of-Action Evaluation and Human Relevance. Toxicol Sci. Oxford University Press; 2009 Jan 1;111(1):kfp131–18.

186. Wen VW, MacKenzie KL. Modeling human endothelial cell transformation in vascular neoplasias. Disease Models & Mechanisms. The Company of Biologists Ltd; 2013 Sep 1;6(5):1066–79.

187. Krump-Konvalinkova V, Bittinger F, Olert J, Bräuninger W, Brunner J, Kirkpatrick CJ. Establishment and Characterization of an Angiosarcoma-Derived Cell Line, AS-M. Endothelium. Taylor & Francis; 2009 Jul 13;10(6):319–28.

188. Masuzawa M, Fujimura T, Hamada Y. Establishment of a human hemangiosarcoma cell line (ISO-HAS). … journal of cancer. 1999.

189. Hoover ML, Větvička V, Hoffpauir JM, Tamburro CH. Human endothelial cell line from an angiosarcoma. In Vitro Cell Dev Biol - Animal. Springer-Verlag; 1993;29(3):199–202.

190. Masuzawa M, Fujimura T, Tsubokawa M, Nishiyama S, Katsuoka K, Terada E, et al. Establishment of a new murine-phenotypic angiosarcoma cell line (ISOS-1). Journal of Dermatological Science. Elsevier; 1998 Jan 1;16(2):91–8.

191. Unger RE, Krump-Konvalinkova V, Peters K, Kirkpatrick CJ. In Vitro Expression of the Endothelial Phenotype: Comparative Study of Primary Isolated Cells and Cell Lines, Including the Novel Cell Line HPMEC-ST1.6R. Microvascular Research. 2002 Nov;64(3):384–97.

192. Unger RE, Oltrogge JB, Briesen Von H. Isolation and molecular characterization of brain microvascular endothelial cells from human brain tumors. In Vitro Cellular & …. 2002.

193. Haseman JK, Hailey JR, Morris RW. Spontaneous Neoplasm Incidences in Fischer 344 Rats and B6C3F1 Mice in Two-Year Carcinogenicity Studies: A National Toxicology Program Update. Toxicol Pathol. SAGE Publications; 1998 May 1;26(3):428–41.

194. Sato N, Kaku T, Dempo K, Kikuchi K. The relationship of colonic carcinogenesis and hepatic vascular tumors induced by subcutaneously injected-1,2-dimethylhydrazine dihydrochloride in specific pathogen free mice. Cancer Letters. Elsevier; 1984 Sep 1;24(2):213–20.

195. Hong CB, Winston JM, Lee CC. Hepatic angiosarcoma. Animal model: angiosarcoma of rats and mice induced by vinyl chloride. The American Journal of Pathology. American Society for Investigative Pathology; 1980 Dec;101(3):737–40.

196. Boivin-Angèle S, Lefrançois L, Froment O, Spiethoff A, Bogdanffy MS, Wegener K, et al. ras gene mutations in vinyl chloride-induced liver tumours are carcinogen-specific but vary with cell type and species. International Journal of Cancer. John Wiley & Sons, Inc; 2000 Jan 15;85(2):223–7.

197. Hong H-HL, Devereux TR, Melnick RL, Moomaw CR, Boorman GA, Sills RC. Mutations of ras Protooncogenes and p53 Tumor Suppressor Gene in Cardiac Hemangiosarcomas from B6C3F1 Mice Exposed to 1,3-Butadiene for 2 Years. Toxicol Pathol. SAGE Publications; 2000 Jul 1;28(4):529–34.

198. Landuzzi L, Ianzano ML, Nicoletti G, Palladini A, Grosso V, Ranieri D, et al. Genetic prevention of lymphoma in p53 knockout mice allows the early development of p53-related sarcomas. Oncotarget. Impact Journals; 2014 Dec 15;5(23):11924–38.

199. Papa A, Cordon-Cardo C, Bernardi R, Pandolfi PP. Compound In Vivo Inactivation of Pml and p53 Uncovers a Functional Interaction in Angiosarcoma Suppression. Genes Cancer. SAGE Publications; 2012 Sep;3(9-10):599–603.

200. Zindy F, Nilsson LM, Nguyen L, Meunier C, Smeyne RJ, Rehg JE, et al. Hemangiosarcomas, medulloblastomas, and other tumors in Ink4c/p53-null mice. Cancer Res. American Association for Cancer Research; 2003 Sep 1;63(17):5420–7.

201. Yang J, Kantrow S, Sai J, Hawkins OE, Boothby M, Ayers GD, et al. Ikk4a/Arf Inactivation with Activation of the NF-κB/IL-6 Pathway Is Sufficient to Drive the Development and Growth of Angiosarcoma. Cancer Res. American Association for Cancer Research; 2012 Sep 15;72(18):4682–95.

202. Leech JD, Lammers SHT, Goldman S, Auricchio N, Bronson RT, Kwiatkowski DJ, et al. A Vascular Model of Tsc1 Deficiency Accelerates Renal Tumor Formation with Accompanying Hemangiosarcomas. Mol Cancer Res. American Association for Cancer Research; 2015 Mar 1;13(3):548–55.

203. Kwiatkowski DJ, Zhang H, Bandura JL, Heiberger KM, Glogauer M, el-Hashemite N, et al. A mouse model of TSC1 reveals sex-dependent lethality from liver hemangiomas, and up-regulation of p70S6 kinase activity in Tsc1 null cells. Human Molecular Genetics. Oxford University Press; 2002 Mar 1;11(5):525–34.

204. Sun S, Chen S, Liu F, Wu H, McHugh J, Bergin IL, et al. Constitutive Activation of mTORC1 in Endothelial Cells Leads to the Development and Progression of Lymphangiosarcoma through VEGF Autocrine Signaling. Cancer Cell. 2015 Dec;28(6):758–72.

205. Dill MT, Rothweiler S, Djonov V, Hlushchuk R, Tornillo L, Terracciano L, et al. Disruption of Notch1 Induces Vascular Remodeling, Intussusceptive Angiogenesis, and Angiosarcomas in Livers of Mice. Gastroenterology. 2012 Apr;142(4):967–977.e2.

206. Rothweiler S, Dill MT, Terracciano L, Makowska Z, Quagliata L, Hlushchuk R, et al. Generation of a murine hepatic angiosarcoma cell line and reproducible mouse tumor model. Lab Invest. Nature Publishing Group; 2015 Mar 1;95(3):351–62.

207. Meis-Kindblom JM, Kindblom LG. Angiosarcoma of soft tissue: a study of 80 cases. The American Journal of Surgical Pathology. 1998 Jun;22(6):683–97.

208. Abraham JA, Hornicek FJ, Kaufman AM, Harmon DC, Springfield DS, Raskin KA, et al. Treatment and outcome of 82 patients with angiosarcoma. Ann Surg Oncol. Springer-Verlag; 2007 Jun;14(6):1953–67.

209. Fury MG, Antonescu CR, Van Zee KJ, Brennan ME, Maki RG. A 14-Year Retrospective Review of Angiosarcoma. The Cancer Journal. 2005 May;11(3):241–7.

210. Fayette J, Martin E, Piperno-Neumann S, Le Cesne A, Robert C, Bonvalot S, et al. Angiosarcomas, a heterogeneous group of sarcomas with specific behavior depending on primary site: a retrospective study of 161 cases. Ann Oncol. Oxford University Press; 2007 Jan 1;18(12):mdm381–2036.

211. Lahat G, Dhuka AR, Hallevi H, Xiao L, Zou C, Smith KD, et al. Angiosarcoma: clinical and molecular insights. Ann Surg. 2010 Jun;251(6):1098–106.

212. Sinnamon AJ, Neuwirth MG, McMillan MT, Ecker BL, Bartlett EK, Zhang PJ, et al. A prognostic model for resectable soft tissue and cutaneous angiosarcoma. Journal of Surgical Oncology. 2016 Oct;114(5):557–63.

213. Pawlik TM, Paulino AF, Mcginn CJ, Baker LH, Cohen DS, Morris JS, et al. Cutaneous angiosarcoma of the scalp. Cancer. Wiley Subscription Services, Inc., A Wiley Company; 2003 Oct 15;98(8):1716–26.

214. Italiano A, Cioffi A, Penel N, Levra MG, Delcambre C, Kalbacher E, et al. Comparison of doxorubicin and weekly paclitaxel efficacy in metastatic angiosarcomas. Cancer. Wiley Subscription Services, Inc., A Wiley Company; 2011 Nov 1;118(13):3330–6.

215. Penel N, Italiano A, Ray-Coquard I, Chaigneau L, Delcambre C, Robin YM, et al. Metastatic angiosarcomas: doxorubicin-based regimens, weekly paclitaxel and metastasectomy significantly improve the outcome. Ann Oncol. Oxford University Press; 2012 Feb;23(2):517–23.

216. Van Glabbeke M, Van Oosterom AT, Oosterhuis JW, Mouridsen H, Crowther D, Somers R, et al. Prognostic Factors for the Outcome of Chemotherapy in Advanced Soft Tissue Sarcoma: An Analysis of 2,185 Patients Treated With Anthracycline-Containing First-Line Regimens—A European Organization for Research and Treatment of Cancer Soft Tissue and Bone Sarcoma Group Study. Journal of Clinical Oncology. American Society of Clinical Oncology; 2016 Sep 21.

217. Asmane I, Litique V, Heymann S, Marcellin L, Métivier A-C, Duclos B, et al. Adriamycin, cisplatin, ifosfamide and paclitaxel combination as front-line chemotherapy for locally advanced and metastatic angiosarcoma. Analysis of three case reports and review of the literature. Anticancer Res. 2008 Sep;28(5B):3041–5.

218. Stacchiotti S, Palassini E, Sanfilippo R, Vincenzi B, Arena MG, Bochicchio AM, et al. Gemcitabine in advanced angiosarcoma: a retrospective case series analysis from the Italian Rare Cancer Network. Ann Oncol. Oxford University Press; 2012 Feb;23(2):501–8.

219. Luo Z-G, Wang Q, Peng W, Hu X-C, Hong X-N. Advanced breast angiosarcoma completely responding to gemcitabine-containing chemotherapy. Breast Care (Basel). 2012 Oct;7(5):414–6.

220. Arnaout A, Wedman DM, El-Sayed S, Acharya V, Lad S. Neoadjuvant Gemcitabine-Taxane Chemotherapy for Radiation-Induced Angiosarcoma of the Breast: A Case Report. The Breast Journal. Blackwell Publishing Ltd; 2012 Apr 5;18(3):276–8.

221. Schlemmer M, Reichardt P, Verweij J, Hartmann JT, Judson I, Thyss A, et al. Paclitaxel in patients with advanced angiosarcomas of soft tissue: a retrospective study of the EORTC soft tissue and bone sarcoma group. Eur J Cancer. Elsevier; 2008 Nov;44(16):2433–6.

222. Penel N, Bui BN, Bay J-O, Cupissol D, Ray-Coquard I, Piperno-Neumann S, et al. Phase II trial of weekly paclitaxel for unresectable angiosarcoma: the ANGIOTAX Study. J Clin Oncol. American Society of Clinical Oncology; 2008 Nov 10;26(32):5269–74.

223. Hirata T, Yonemori K, Ando M, Hirakawa A, Tsuda H, Hasegawa T, et al. Efficacy of taxane regimens in patients with metastatic angiosarcoma. Eur J Dermatol. 2011 Jul;21(4):539–45.

224. Ye J, Li XF, Wang YD, Yuan Y. Long‐term survival of a patient with scalp angiosarcoma and multiple metastases treated using combination therapy: A case report. Oncology Letters. Spandidos Publications; 2015 Jan 29;9(4):1725–8.

225. Kiesel H, Müller AMS, Schmitt-Graeff A, Veelken H. Dramatic and durable efficacy of imatinib in an advanced angiosarcoma without detectable KIT and PDGFRA mutations. Cancer Biol Ther. 2009 Feb;8(4):319–21.

226. Kanat O, Ozet A, Ataergin SA, Ozturk B, Arpaci F, Ozturk M, et al. A Case of Advanced Scalp Angiosarcoma Successfully Treated with Combination Chemotherapy of Adriamycin, Cisplatin and Ifosfamide. Clinical Oncology. Clinical …; 2006 Jun;18(5):426–7.

227. Mathew P, Vakar-Lopez F, Troncoso P. Protracted remission of metastatic epithelioid angiosarcoma with weekly infusion of doxorubicin, paclitaxel, and cisplatin. The Lancet Oncology. Elsevier; 2006 Jan;7(1):92–3.

228. Kopp H-G, Kanz L, Hartmann JT. Complete remission of relapsing high-grade angiosarcoma with single-agent metronomic trofosfamide. Anticancer Drugs. 2006 Sep;17(8):997–8.

229. Mocellin S, Rossi CR, Brandes A, Nitti D. Adult soft tissue sarcomas: conventional therapies and molecularly targeted approaches. Cancer Treatment Reviews. Elsevier; 2006 Feb;32(1):9–27.

230. Kasper B, Gil T, Awada A. Treatment of patients with advanced soft tissue sarcoma: disappointment or challenge? Current Opinion in Oncology. 2007 Jul;19(4):336–40.

231. Ferrara N, Kerbel RS. Angiogenesis as a therapeutic target. Nature. Nature Publishing Group; 2005 Dec 15;438(7070):967–74.

232. Maki RG, D'Adamo DR, Keohan ML, Saulle M, Schuetze SM, Undevia SD, et al. Phase II study of sorafenib in patients with metastatic or recurrent sarcomas. J Clin Oncol. American Society of Clinical Oncology; 2009 Jul 1;27(19):3133–40.

233. Park MS, Ravi V, Araujo DM. Inhibiting the VEGF–VEGFR pathway in angiosarcoma, epithelioid hemangioendothelioma, and hemangiopericytoma/solitary fibrous tumor. Current Opinion in Oncology. 2010 Jul 1;22(4):351–5.

234. Agulnik M, Okuno SH, Mehren Von M, Jovanovic B. An open-label multicenter phase II study of bevacizumab for the treatment of angiosarcoma. ASCO Annual Meeting …; 2009.

235. George S, Merriam P, Maki RG, Van den Abbeele AD, Yap JT, Akhurst T, et al. Multicenter phase II trial of sunitinib in the treatment of nongastrointestinal stromal tumor sarcomas. J Clin Oncol. American Society of Clinical Oncology; 2009 Jul 1;27(19):3154–60.

236. Medici D, Olsen BR. Rapamycin Inhibits Proliferation of Hemangioma Endothelial Cells by Reducing HIF-1-Dependent Expression of VEGF. Lebedeva IV, editor. PLoS ONE. Public Library of Science; 2012 Oct 8;7(8):e42913.

237. Carvajal RD, Tse A, Schwartz GK. Aurora Kinases: New Targets for Cancer Therapy. Clin Cancer Res. American Association for Cancer Research; 2006 Dec 1;12(23):6869–75.

238. Graff JN, Higano CS, Hahn NM, Taylor MH, Zhang B, Zhou X, et al. Open-label, multicenter, phase 1 study of alisertib (MLN8237), an aurora A kinase inhibitor, with docetaxel in patients with solid tumors. Cancer. 2016 Aug 15;122(16):2524–33.

239. Ashley EA, Butte AJ, Wheeler MT, Chen R, Klein TE, Dewey FE, et al. Clinical assessment incorporating a personal genome. The Lancet. 2010 May;375(9725):1525–35.

240. Simon R, Roychowdhury S. Implementing personalized cancer genomics in clinical trials. Nature Reviews Drug Discovery. Nature Publishing Group; 2013 May 1;12(5):358–69.

241. Dow LE, Fisher J, O'Rourke KP, Muley A, Kastenhuber ER, Livshits G, et al. Inducible in vivo genome editing with CRISPR-Cas9. Nature Biotechnology. Nature Research; 2015 Apr 1;33(4):390–4.

242. Albers J, Danzer C, Rechsteiner M, Lehmann H, Brandt LP, Hejhal T, et al. A versatile modular vector system for rapid combinatorial mammalian genetics. J Clin Invest. American Society for Clinical Investigation; 2015 Apr;125(4):1603–19.

243. Katzen F. Gateway(®) recombinational cloning: a biological operating system. Expert Opin Drug Discov. 2007 Apr;2(4):571–89.

244. Schambach A, Galla M, Modlich U, Will E, Chandra S, Reeves L, et al. Lentiviral vectors pseudotyped with murine ecotropic envelope: increased biosafety and convenience in preclinical research. Exp Hematol. Elsevier; 2006 May;34(5):588–92.

245. Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell. 1997 Mar 7;88(5):593–602.

246. Davies BR, Logie A, McKay JS, Martin P, Steele S, Jenkins R, et al. AZD6244 (ARRY-142886), a potent inhibitor of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1/2 kinases: mechanism of action in vivo, pharmacokinetic/pharmacodynamic relationship, and potential for combination in preclinical models. Mol Cancer Ther. American Association for Cancer Research; 2007 Aug 1;6(8):2209–19.

247. Maira S-M, Stauffer F, Brueggen J, Furet P, Schnell C, Fritsch C, et al. Identification and characterization of NVP-BEZ235, a new orally available dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor with potent in vivo antitumor activity. Mol Cancer Ther. American Association for Cancer Research; 2008 Jul;7(7):1851–63.

248. Engelman JA, Chen L, Tan X, Crosby K, Guimaraes AR, Upadhyay R, et al. Effective use of PI3K and MEK inhibitors to treat mutant Kras G12D and PIK3CA H1047R murine lung cancers. Nature Medicine. Nature Publishing Group; 2008 Nov 30;14(12):1351–6.

249. Vichai V, Kirtikara K. Sulforhodamine B colorimetric assay for cytotoxicity screening. Nature Protocols. 2006;1(3):1112–6.

250. Xue W, Chen S, Yin H, Tammela T, Papagiannakopoulos T, Joshi NS, et al. CRISPR-mediated direct mutation of cancer genes in the mouse liver. Nature. Nature Research; 2014 Oct 16;514(7522):380–4.

251. Maddalo D, Manchado E, Concepcion CP, Bonetti C, Vidigal JA, Han Y-C, et al. In vivo engineering of oncogenic chromosomal rearrangements with the CRISPR/Cas9 system. Nature. Nature Research; 2014 Dec 18;516(7531):423–7.

252. Platt RJ, Chen S, Zhou Y, Yim MJ, Swiech L, Kempton HR, et al. CRISPR-Cas9 Knockin Mice for Genome Editing and Cancer Modeling. Cell. 2014 Oct;159(2):440–55.

253. Sigmund CD. Viewpoint: are studies in genetically altered mice out of control? Arterioscler Thromb Vasc Biol. 2000 Jun;20(6):1425–9.

254. Wilson C. A mouse model of tuberous sclerosis 1 showing background specific early post-natal mortality and metastatic renal cell carcinoma. Human Molecular Genetics. Oxford University Press; 2005 May 11;14(13):1839–50.

255. Medina D. Mammary tumorigenesis in chemical carcinogen-treated mice. I. Incidence in BALB-c and C57BL mice. J Natl Cancer Inst. 1974 Jul;53(1):213–21.

256. Ullrich RL, Bowles ND, Satterfield LC, Davis CM. Strain-dependent susceptibility to radiation-induced mammary cancer is a result of differences in epithelial cell sensitivity to transformation. Radiat Res. Radiation Research Society; 1996 Sep;146(3):353–5.

257. Zhang S, Qian X, Redman C, Bliskovski V, Ramsay ES, Lowy DR, et al. p16INK4a gene promoter variation and differential binding of a repressor, the ras-responsive zinc-finger transcription factor, RREB. Oncogene. 2003 Apr 17;22(15):2285–95.

258. Blackburn AC, Brown JS, Naber SP, Otis CN, Wood JT, Jerry DJ. BALB/c alleles for Prkdc and Cdkn2a interact to modify tumor susceptibility in Trp53+/- mice. Cancer Res. 2003 May 15;63(10):2364–8.

259. Rodriguez R, Rubio R, Menendez P. Modeling sarcomagenesis using multipotent mesenchymal stem cells. Cell Research. Nature Publishing Group; 2011 Sep 20;22(1):62–77.

260. Rubio R, García-Castro J, Gutiérrez-Aranda I, Paramio J, Santos M, Catalina P, et al. Deficiency in p53 but not Retinoblastoma Induces the Transformation of Mesenchymal Stem Cells In vitro and Initiates Leiomyosarcoma In vivo. Cancer Res. American Association for Cancer Research; 2010 May 12;70(10):4185–94.

261. Tolar J, Nauta AJ, Osborn MJ, Panoskaltsis Mortari A, McElmurry RT, Bell S, et al. Sarcoma derived from cultured mesenchymal stem cells. Stem Cells. John Wiley & Sons, Ltd; 2007 Feb;25(2):371–9.

262. Guarnerio J, Riccardi L, Taulli R, Maeda T, Wang G, Hobbs RM, et al. A genetic platform to model sarcomagenesis from primary adult mesenchymal stem cells. Cancer Discov. American Association for Cancer Research; 2015 Apr;5(4):396–409.

263. He Q, Wan C, Li G. Concise review: multipotent mesenchymal stromal cells in blood. Stem Cells. John Wiley & Sons, Ltd; 2007 Jan;25(1):69–77.

264. Johnson TE, Umbenhauer DR, Hill R, Bradt C, Mueller SN, Levine EM, et al. Karyotypic and phenotypic changes during in vitro aging of human endothelial cells. Journal of Cellular Physiology. Wiley Subscription Services, Inc., A Wiley Company; 1992 Jan 1;150(1):17–27.

265. Wagner M, Hampel B, Bernhard D, Hala M, Zwerschke W, Jansen-Dürr P. Replicative senescence of human endothelial cells in vitro involves G1 arrest, polyploidization and senescence-associated apoptosis. Experimental Gerontology. 2001 Aug;36(8):1327–47.

266. Rudolph KL, Millard M, Bosenberg MW, DePinho RA. Telomere dysfunction and evolution of intestinal carcinoma in mice and humans. Nat Genet. Nature Publishing Group; 2001 Jun 1;28(2):155–9.

267. Wan X, Harkavy B, Shen N, Grohar P, Helman LJ. Rapamycin induces feedback activation of Akt signaling through an IGF-1R-dependent mechanism. Oncogene. Nature Publishing Group; 2007 Mar 22;26(13):1932–40.

268. Chawla SP, Staddon AP, Baker LH, Schuetze SM, Tolcher AW, D'Amato GZ, et al. Phase II Study of the Mammalian Target of Rapamycin Inhibitor Ridaforolimus in Patients With Advanced Bone and Soft Tissue Sarcomas. Journal of Clinical Oncology. American Society of Clinical Oncology; 2011 Dec 29;30(1):78–84.

269. Andersen NJ, Boguslawski EB, Kuk CY, Chambers CM, Duesbery NS. Combined inhibition of MEK and mTOR has a synergic effect on angiosarcoma tumorgrafts. Int J Oncol. Spandidos Publications; 2015 Jul;47(1):71–80.

270. Weissleder R. A clearer vision for in vivo imaging. Nature Biotechnology. 2001 Apr;19(4):316-7.

271. Morgan RA, Nussbaum O, Muenchau DD, Shu L, Couture L, Anderson WF. Analysis of the functional and host range-determining regions of the murine ectropic and amphotropic retrovirus envelope proteins. J Virol. American Society for Microbiology; 1993 Aug;67(8):4712–21.

272. Kim JW, Closs EI, Albritton LM, Cunningham JM. Transport of cationic amino acids by the mouse ecotropic retrovirus receptor. Nature. Nature Publishing Group; 1991 Aug 22;352(6337):725–8.

273. Proteinatlas SLC7A1 tissue expression. http://www.proteinatlas.org/ENSG00000139514-SLC7A1/tissue.

274. Hines WC, Yaswen P, Bissell MJ. Modelling breast cancer requires identification and correction of a critical cell lineage-dependent transduction bias. Nat Commun. Nature Publishing Group; 2015 Apr 21;6:6927.

275. Arcasoy SM, Latoche J, Gondor M, Watkins SC, Henderson RA, Hughey R, et al. MUC1 and Other Sialoglycoconjugates Inhibit Adenovirus-mediated Gene Transfer to Epithelial Cells. American Journal of Respiratory Cell and Molecular Biology. American Thoracic Society New York, NY; 1997 Oct;17(4):422–35.

LAURA PATRICIA BRANDT

Born 31.05.1988 in Hamburg, Germany

EDUCATION

Doctor of Philosophy in Cancer Biology November 2012 - current
University of Zurich, Institute of Physiology
Cancer Biology PhD program/ LSZGS
Title: Modelling soft tissue sarcomas using somatic mouse genetics
Supervisor: Prof. Dr. Ian J. Frew

Master of Science in Molecular Medicine October 2011 - September 2012
Imperial College London, Department of Surgery and Cancer
Title: Functional studies of cancer associated mutations in the liver receptor homolog-1 transcription factor
Supervisors: Prof. Dr. Simak Ali and Prof. Dr. Lakjaya Buluwela

Bachelor of Science in Molecular Life Sciences October 2008 - September 2011
University of Hamburg, Institute for Biochemistry
Title: Mechanismus der zellulären Aufnahme von Inositolhexakisphosphat in Tumorzellen
Supervisors: Prof. Dr. Georg W. Mayr and Dr. Sabine Windhorst

Allgemeine Hochschulreife 1998 - 2007
Gymnasium Buckhorn, Hamburg, Germany

PUBLICATIONS

A versatile modular vector system for rapid combinatorial mammalian genetics. Albers J, Danzer C, Rechsteiner M, Lehmann H, Brandt LP, Hejhal T, Catalano A, Busenhart P, Gonçalves AF, Brandt S, Bode PK, Bode-Lesniewska B, Wild PJ, Frew IJ. Journal of Clinical Investigations, 2015, 125(4):1603-19.

Tumour cells can employ extracellular Ins(1,2,3,4,5,6)P(6) and multiple inositol-polyphosphate phosphatase 1 (MINPP1) dephosphorylation to improve their proliferation. Windhorst S, Lin H, Blechner C, Fanick W, Brandt L, Brehm MA, Mayr GW. Biochemical Journal, 2013, 450(1):115-25.